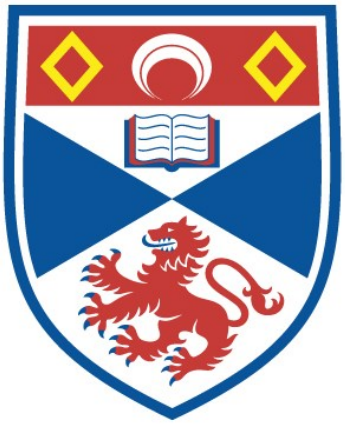
STUDIES OF CRUSTACEAN HYPERGLYCAEMIC HORMONE IN THE NORWAY LOBSTER 'NEPHROPS NORVEGICUS' (LINNAEUS, 1758)

Richard Paul Smullen

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1993

Studies of Crustacean Hyperglycaemic Hormone in the Norway Lobster *Nephrops norvegicus* (Linnaeus, 1758).

by

Richard Paul Smullen

A thesis submitted for the degree of Doctor of Philosophy in the University of St. Andrews.

10th May 1993.

Nephrops norvegicus.

To Mum, Dad and Mark.

Declaration

a) I, Richard Paul Smullen, hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial / complete fulfillment of any other degree or qualification.

Signed Date 10th MAY 1993

b) I was admitted to the Faculty of Science of the University of St. Andrews, under Ordinance General No. 12 on 1st October 1989 and as a candidate for the degree of Ph. D. on 1st October 1990.

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c) I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the Degree of Ph. D.

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Ode to a lobster.

There was a time, many years ago, when lobsters, like all creatures great and small, walked in a forward direction across the ocean floor. Today, as most of you know, lobsters spend more time walking backward. "How," you might ask, "could such a thing come about?" Well the answer, my friends, is simply these poor creatures have been poked and prodded, studied, examined and tested by so many scientists for so many years they have learned to walk this way the better to see and avoid the ever–present biologist.

Unknown author, from Aiken, D.E., (1977).

Acknowledgements.

I would like to take this opportunity to thank my supervisor, Dr. Matthew Bentley. Without his pearls of wisdom, his never ending encouragement and patience, and his ability to calm my ragged nerves, I would have surely never completed this thesis. His sense of humour, although at times intolerably dry, kept me going.

Many thanks also go to Professor Dr. R. Keller, from the University of Bonn for the donation of anti-*Orconectes* CHH antisera, to Dr. D. Soyez, from the Ecole Normale Supérieure, Paris, for the gift of anti-*Homarus* GIH antisera and to Professor F. Van Herp from the Catholic University, Nijmegen, for the donation of the oligonucleotide primers. In addition, the hospitality shown to me by Professor Keller, Dr. Soyez and Professor Van Herp and the members of their research teams on my visit to their laboratories was greatly appreciated. Thanks must also be extended to Professor Spindler and his wife for their hospitality while at the University of Düsseldorf and individually to Dr. R. Lafont, Dr. H. Dircksen, Dr. S. Webster, Kees Tensen and Domenique de Kleijn for all their help and advise.

Many thanks to Dr. G. Cramb, around whose molecular biological laboratory, I was allowed to run riot without the use of a blank cheque book and to his staff and research students, in particular Ian Sanders and Dr. Chris Cutler, who have tried in vain to turn me into a molecular biologist. In addition, I would like to extend my thanks Dr. Tony Crockford for his help with the SDS-PAGE and the capillary electrophoresis, to Zandra Horne for her assistance with the ELISA and antibody work and to Dr. J. Michael. Conlon, at the University of Creighton, Nebraska for sequencing my peptide samples. I am also indebted to Dr. R. Hay who kindly donated the Protein A Sepharose, to Mr. R. Stuart who provided the histological expertise and to Sean Earnshaw, Karen Johnson and Dave Roche for the processing of the photographic material contained within this thesis. Thanks to Alan Hill at The Gleneagles Hotel, Auchterarder, and to Tony Binks at the Caledonian Hotel, Edinburgh for allowing the collection of *Nephrops norvegicus* eyestalks and to Tom Gordon and the crew of the "Twa Gordons" who collected the live animals that were used during this study.

Many thanks must go to my proof readers, Iain Johnson, Dr. Derek Ball, Helen Jones and Dave Matthews, to Dr. C. Todd, Dr. N. Hazon and Professor M. Laverack for all their help and advice during my time at the Gatty, to Jane Williamson for putting up with me using her office and equipment and to Margaret Robertson for her rolls. In retrospect, I should thank just about everyone who has been at the Gatty during my stay here and made the place feel like a home from home.

Finally, huge thanks must go to Mum and Dad who have always been there to talk to and to Julie who has put up with so much hassle and listened to so much twaddle.

Abstract.

Nephrops norvegicus is a deep water marine decapod crustacean which burrows in fine muddy substrata. It is a commercially important as a fisheries species, but knowledge of its biology, particularly concerning its endocrinology, is limited. This thesis describes the endocrinology of *Nephrops norvegicus* with particular reference to crustacean hyperglycaemic hormone.

Histological investigations of the eyestalk of *Nephrops norvegicus* enabled the identification of the X-organ sinus gland complex. The development of a micro-bioassay allowed the determination of increases of glycaemia following the injection of crude sinus gland extracts into a host animal. The optimal dose for induced haemolymph glycaemia was determined.

HPLC separation was used to isolate and purify several neuropeptides from crude sinus gland extracts, which had varying degrees of hyperglycaemic activities. The use of ELISA (enzyme linked immunosorbent assay) showed that the peptides reacted with polyclonal rabbit antiserum raised against the CHH of the crayfish *Orconectes limosus* and with polyclonal guinea pig antiserum raised against the GIH of the lobster, *Homarus americanus*. SDS-PAGE of these peptides enabled an estimation of their molecular weights and the purity of one of the active peptides was determined using capillary electrophoresis.

The effects of photoperiod and severe hypoxia on the CHH-induced hyperglycaemia of *Nephrops norvegicus* were also investigated. The responses of *N. norvegicus* appeared to differ in some respects from other decapods species.

Pairs of oligonucleotide primers, based on the sequence of the lobster, *Homarus americanus,* complimentary to regions of the CHH sequence, were used in the polymerase chain reactions (PCR). Complimentary first strand DNA (cDNA) was synthesised from total *Nephrops* eyestalk RNA. 35 rounds and 40 rounds of PCR amplification produced a 100bp and 230bp double stranded DNA product respectively, which were resolved by electrophoresis on a tris-borate-EDTA gel. The product size was compared with known standards.

Finally, the identification of CHH and GIH synthesis and storage in the eggs of *Nephrops norvegicus* at various stages of development was investigated. By the use of PCR, it was not possible to determine if synthesis of CHH was occurring in 50% developed eggs, The use of ELISA, however, demonstrated that in 90% developed eggs there was a significant increase of both CHH and GIH immunoactivity.

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List of abbreviations.

5-HT	serotonin
AC	adenylate cyclase
αdCTP	α deoxy cytosine triphosphate
AKH	adipokinetic hormone
APRP	adipokinetic hormone precursor related peptide
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
CCAP	crustacean cardioactive peptide
cDNA	complimentary deoxyribonucleic acid
CE	capillary electrophoresis
cGMP	cyclic guanosine monophosphate
CHH	crustacean hyperglycaemic hormone
CHH B-RI	crustacean hyperglycaemic hormone retained intron
CNDPK	cyclic nucleotide dependent protein kinase
CNS	central nervous system
COC	circumoesophageal commissures
CPRP	crustacean hyperglycaemic hormone precursor related peptide
cRNA	complimentary ribonucleic acid
DAS-ELISA	double antibody sandwich enzyme linked immunosorbent assay
DEP	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dNTPS	deoxy Nucleotide Tri Phosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
FAB/MS	fast atom bombardment/mass spectrometry
G/CCK	gastrin cholecystokinin
GC	guanylate cyclase
GIH	gonad inhibiting hormone
GTP	guanosine triphophate
H_2O_2	hydrogen peroxide
HPLC	high performance liquid chromatography
MF	methyl farnosoate
MIH	moult inhibiting hormone
MOPS	3-(N-Morpholino)propanesulfonic acid

mRNA	messenger ribonucleic acid
MTGXO	medulla terminalis ganglionic X-organ
MTGXO-SG	medulla terminalis ganglionic X-organ-sinus gland complex
P1	primer 1
P2	primer 2
P3	primer 3
PAF	paraldhyde fuchsin
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCO	post-commissural organ
PCR	polymerase chain reaction
PDH	pigment dispersing hormone
PGO	peroxidase/glucose oxidase
PO	pericardial organ
RIA	radioimmunoassay
RNA	ribonucleic acid
RNase	ribonuclease
RP HPLC	reverse phase high performance liquid chromatography
RPCH	red pigment concentrating hormone
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TFA	trifluoroacetic acid
TFSSW	twice filtered sterile sea water
TFSW	twice filtered sea water
TRIS	tris(hydroxymethyl)aminomethane
tRNA	total ribonucleic acid
TWEEN	polyoxyethylenesorbitan
VIH	vitellogenesis inhibiting hormone
XOSG	X-organ sinus gland

Chapter 1.

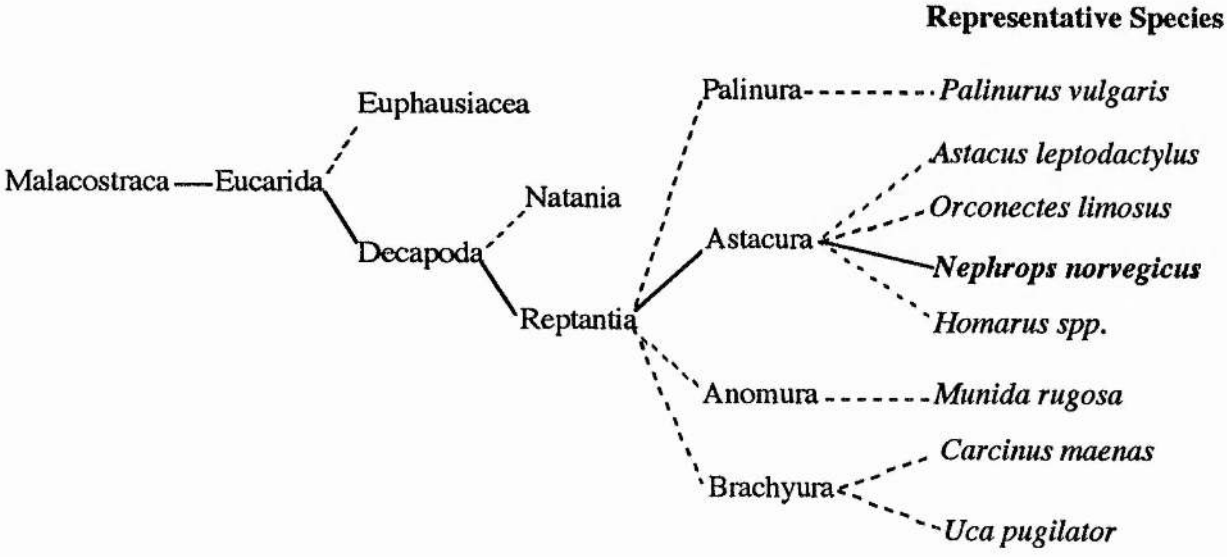
Introduction.

The biology of *Nephrops norvegicus* and an introduction to crustacean endocrinology.

1.1 Biology of *Nephrops norvegicus*.

1.1.1 Nomenclature.

The species was first named *Nephrops norvegicus* by Linnaeus, 1758 and was re-named by subsequent researchers to: *Cancer norvegicus* Linnaeus, 1758; *Astacus norvegicus* (Linnaeus, 1758) Fabricius, 1775; *Astacus norvegicus* (Linnaeus, 1758) Pennant, 1777; *Homarus norvegicus* (Linnaeus, 1758) Weber, 1795; *Nephrops norvegicus* (Linnaeus, 1758) Leach, 1814; *Nephrops norvegicus meridionalis* (Linnaeus, 1758) Zariquiey Cenarro, 1935 (see Farmer, 1975). All existing species ascribed to this genus other than *Nephrops norvegicus*, have been removed by Jenkins (1972) and given to the genus *Metanephrops*. The differences between these two genera are reviewed by Farmer (1975) from Jenkins (1972). The valid species name is *Nephrops norvegicus* (Linnaeus, 1758) and the species taxonomy and the relationship to other Crustacea is described below.



1.1.2 Distribution

Nephrops norvegicus (Linnaeus, 1758) is a marine boreal crustacean species which is widely distributed on the continental shelf of the north-east Atlantic, with its limits stretching as far north as Greenland/Iceland and as far south and east as Morocco and Egypt. The species is located primarily throughout Scottish and western Irish waters, extending through the North Sea to the Danish coasts and the Kattegat, the entrance to the Baltic Sea, although the animal is not fished commercially within the Baltic Sea itself. Additional large concentrations of *Nephrops* are situated off the western coast of France stretching throughout the western Mediterranean and becoming less widespread eastward (Farmer, 1975). It should be noted that our present knowledge of the population structure, life cycle and general ecology of *Nephrops* are derived largely from the analysis of catch data from commercial and research fishing vessels. As *Nephrops* can only be caught when they emerge from their burrows, this data can, therefore, only give us a partial representation of the animal's ecology and thus should be taken into account when interpreting ecological data (Chapman, 1980). In the U.K. in 1986, commercial fishery landings of *Nephrops* totalled 25,515 tonnes with a market value of £39 million (Thompson and Ayers, 1989).

Nephrops norvegicus are benthic animals which construct burrows in the soft bottom sediment. Although the 3 stages of larvae are planktonic, the greater part of the animal's life is spent within the confines of the burrow which functions as a refuge. When observing population distribution and density of *Nephrops*, sediment type is a major governing factor and it is this selectivity of suitable burrowing substrata that leads to the discontinuous distribution of *Nephrops* throughout north-east Europe. The mean size of individuals is positively correlated to the silt-clay content of the sediment (Chapman and Bailey, 1986) where between 50% and 85% of *Nephrops* beds have a particle size of between

0.5μm and 60μm (Rice and Chapman, 1971; Farmer, 1972, 1975). Additionally, the cohesive properties of the sediment are also important for the burrowing behaviour of the animal as the thixotropic nature of the substratum greatly enhances burrowing activity. (Rice and Chapman, 1971; Chapman, 1980).

1.1.3 Differential distribution.

1.1.3.1 Larvae

The larvae of *Nephrops norvegicus*, are released from the eggs carried on the abdomen of the female into the water column from the areas of fine mud inhabited by the adult. They spend approximately two to three weeks in the pelagic planktonic phase where they undergo three larval moults before metamorphosing and settling onto the mud as a juvenile. It is the specific nature of the substratum requirment, that determines successful larval settlement. This in turn influences the distribution of the species and may be an important constraint on the extent of recruitment (White *et al*, 1988). In addition, there appears to be a strong density dependent mortality operating at the juvenile settling stage. Many more larvae will attempt to settle on a suitable mud area than can be accommodated and so currents, advection and horizontal turbulent diffusion will spread larvae away from these areas and in so doing create a dispersal pattern and potentially limit recruitment (Hill, 1990; Hill and White 1990). An advection-diffusion-mortality model has been postulated by Hill (1990) who suggests that losses due to pelagic dispersal are dependent on the nature of the turbulence and the size of the suitable settling area available to the *Nephrops*. Turbulent distribution alone will not affect recruitment and hence population on large mud areas, whereas advection can maintain pelagic larvae within the water column, thus preventing settlement.

The settling juveniles initially inhabit existing burrows and do not make their own (Cobb, 1986), however, Crnkovic (1968), showed that post larval stages burrow in mud but the intricacies of the burrows that they create suggest that this action is a means of foraging rather than refuge creation.

Changes in temperature, salinity, depth, sediment structure, oxygen content and reproductive state (Simpson 1965; Thomas and Figueiredo 1965; Hammond and Naylor 1977a; Bagge and Munch-Petersen 1979; Chapman 1980; Hagerman and Uglow 1985; Baden *et al* 1990; Sardà, 1991) all may influence the population distribution, however these environmental and biological changes appear to affect the behavioural nature of individuals and may not be linked to the population as a whole. It should be noted that as the emergent behaviour of *Nephrops* from their burrows alters under varying environmental regimes, this may ultimately affect the catch ability of the animal, thus creating a misleading estimation of its distribution.

Endogenous influences such as biological clocks, and exogenous factors listed above may interact, in addition to the aforementioned physical factors, in such a way that the life cycles of *Nephrops norvegicus* become adapted to each particular habitat manifesting as population variations not only with latitude but in response to local influences (Chapman, 1980; Chapman and Howard, 1988; Sardà, 1991). Whilst the basic pattern of the animal's life cycle can be anticipated to a certain degree (e.g. growth, gonad development, incubation cycle) other factors (substrate, climate, light cycle, temperature, food availability), act contemporaneously on the population, and these may disrupt this pattern of predictability. An overview of these components are discussed in this chapter. However, other more random and often transient influences may assert themselves upon a population, the affects of which are difficult to quantify. Sardà (1991) describes these as random factors in that they are independent of phenotype or local habitat. The type of exploitation to which the stock is subjected, sudden

abnormal variations of climate, and phenomena that are density dependent or the often random possibility of a mating encounter between a "hard" male and a "soft" female are all described as "random factors". Therefore, a percentage of non-synchrony and variability in the life cycle of *Nephrops norvegicus*, may be the result of these influences. It is suggested that they play a major role in the reproductive cycle of the animal and are thought to have a deleterious effect on the success of annual spawning (Sardà, 1991) thus creating both, between and within population variation.

1.1.3.2 Temperature

Temperature does not appear to be a major influence upon the distribution of *Nephrops norvegicus*. This species generally inhabits deep water (15—800m) (Farmer, 1975) and is, therefore, unlikely to experience large fluctuations in temperature. The maximum and minimum temperatures that limit distribution are unknown, although bottom temperatures vary depending on geographical location; 7—13°C in the Irish Sea (Farmer 1972) and 10—15°C in the Adriatic Sea (Karlovac, 1953). In the Ligurian Sea, *Nephrops* live at a relatively constant temperature of approximately 13°C (Orsi-Relini and Relini, 1989). Heldt and Heldt (1931) suggested that *Nephrops* was a Mediterranean species that had extended its northern limit as a result of the influence of the Gulf Stream, although the reverse scenario has been postulated by Lorenz in 1863 (see Farmer, 1975) in that *Nephrops* could be regarded as a glacial relic. Temperature may play a more important role in areas where water is relatively shallow and temperature fluctuations may occur frequently throughout the year. Between the coasts of Denmark and Sweden, a shallow area of water known as the Kattegat supports a large population of *Nephrops* that live on the thermocline and are thus affected by

large temperature fluctuations (as much as 10°C) and seasonal eutrophication (Baden *et al*, 1990), and as a consequence, seasonal reduction in oxygen tension.

1.1.3.3 Oxygen.

When the water temperature is at its seasonal maximum, oxygen tension and, therefore oxygen availability are at their lowest. During these periods there is often an increase in commercial landings of *Nephrops norvegicus* (Bagge and Munch-Peterson, 1979). In the Kattegat, such low oxygen tensions occur during the summer months, especially between August and November (Hagerman and Uglow, 1985; Baden *et al*, 1990). This increase in catches may be explained by the fact that hypoxic conditions stimulate an emergence behaviour of the *Nephrops*. This movement away from the burrow, makes the animal more susceptible to capture by trawling, and, as a consequence, leads to high numbers of animals landed. This large catch may be disproportionately skewed towards a single sex, typically approximately 78% males. In 1988, however, there was a predominance of females (c. 75%) (Baden *et al*, 1990), although this effect has not been conclusively demonstrated as a result of long term hypoxia.

Under normal conditions *Nephrops norvegicus* may remain within its burrow for many hours, with periods of emergence being restricted to particular times of the day. This burrowing behaviour by *Nephrops* subjects the animal to varying degrees of hypoxia. Other species of burrowing decapods have been shown to tolerate and adapt to low oxygen tensions by the ability to maintain respiration, independent of external oxygen tensions, down to a lower critical level, or to maintain low oxygen consumption rates (Bridges and Brand, 1980). *Nephrops* are able to increase their gill beat frequency during periods of hypoxia, while at the same time the oxygen extraction efficiency of the animal also increases over time (Hagerman and Uglow, 1985). In addition to this, they are capable of increasing the haemocyanin content in the haemolymph during these

periods of hypoxic stress (Baden *et al*, 1990). Behavioural adaptations, such as the circulation of water within the burrow by the beating of the pleopods and of the scaphognathites and the orientation of the burrows allowing passive irrigation by natural water movements (Atkinson and Taylor, 1988), coupled with the metabolic adaptations described above, show that the species is well able to meet the respiratory stresses that low oxygen tensions may exert. However, Hagerman and Uglow (1985) speculated that due to an increase of eutrophication, the benthic fauna, and hence the food supply and quality of the *Nephrops*, was affected by the subsequent hypoxia. This phenomenon was described by Baden *et al* (1990) as hypoxia-induced starvation; the hypoxia causes emergence, although the animals themselves are unable to feed at low oxygen levels, rather than the lack of food hypothesis proposed by Hagerman and Uglow (1985).

1.1.3.4 Light.

There appears to be a connection between emergence of *Nephrops norvegicus* from the burrow and light intensity (Hillis 1971; Chapman *et al* , 1972, 1980; Aréchiga and Atkinson, 1975; Atkinson and Naylor, 1976). Light intensities that are typical with that of shallow water, caused the animals to remain in their burrows even under hypoxia, however when the light intensities reduced, an emergence behaviour was observed (Hagerman and Uglow, 1985). Long term hypoxia did not induce emergent behaviour (Hagerman and Uglow, 1985; Baden *et al*, 1990).

Nephrops norvegicus are most abundant on the substrate surface out of their burrows at night, demonstrating a nocturnally phased, burrow orientated, circadian rhythmicity (Atkinson and Naylor, 1976). The onset of nocturnal burrow emergence in *Nephrops*, occurs at dusk and at a specific irradiance threshold of

$10^{-5}W.m^{-2}$. This activity is irrespective of the rate of onset of darkness (Möller and Naylor, 1980).

Crepuscular activity is displayed by *Nephrops norvegicus* over most of its natural range. Increased depth (150m) however, causes emergence at noon while shallow water (20m) promotes nocturnal emergence. In these situations, changes of light intensity are the main triggers for emergence (Chapman 1980). This may not necessarily correspond to an increased catch rate as the high activity displayed by these animals may permit possible trawl avoidance (Hammond and Naylor, 1977a;b), although increased depth has been shown to shift optimum catch sizes towards the midday period (Atkinson and Naylor, 1976). These periods do not appear to be subject to seasonal variation (Chapman, 1980), although tides and turbidity have been known to affect diurnal catch variations due to attenuation of the light (Chapman *et al*, 1972).

1.1.3.5 Salinity.

Salinity may not have as large an affect on the distribution of *Nephrops norvegicus* populations as some of the aforementioned factors. Höglund (1942) cited by Farmer (1975), however, suggested that *Nephrops* were absent from the Baltic Sea due to the particularly low salinity levels (c. 15‰). Salinities of 38.5‰ have been reported by Orsi-Relini and Relini, (1989) in the Ligurian Sea, although the effects of salinity on *Nephrops* has not been well documented. Sastry and Vargo, (1977) noted that larvae of the lobster, *Homarus americanus*, were more tolerant of salinity variation at higher temperatures, while Templeman, (1936), showed larvae were unable to survive below 17‰.

1.1.3.6 Pollution.

Due to the burrowing behaviour of *Nephrops norvegicus*, the species is highly susceptible to pollution, particularly to the toxic poisoning of the sediment and the smothering effects of sewage. The Clyde estuary is the dumping site for 10^6 tonnes of sewage sludge from Glasgow each year. The dumping of sewage waste started in 1904, when the *Nephrops* fishery did not exist and the actual dump site was on a former *Nephrops* ground (McIntyre and Johnston, 1975). There is a clear boundary where ground containing *Nephrops* burrows changes to black fibrous mud which is unsuitable for burrowing and subsequently larval settlement. This unsuitable area has been estimated to cover approximately $10km^2$. The deleterious effect of eutrophication, and its role in the promotion of long-term hypoxia during the summer months in the Kattegat, due to pollution, have been investigated by Baden *et al,* (1990).

1.1.4 Life history.

There have been a number of review articles that discuss the reproductive strategies and life cycle of *Nephrops norveigcus* (Figueiredo and Thomas, 1967; Farmer, 1974, 1975; Chapman, 1980). These reviews demonstrate the extent of reproductive variations that species appear to display according to their location. For example, in Portuguese waters, egg laying occurs from August to September, with hatching taking place from February to March, an incubation period of 6 months (Figueiredo and Barraca, 1963), whereas animals caught off the north east coast of England, exhibited a later hatch time between May and August, an incubation of 10 months (Symonds, 1972). The female reproductive cycle is further complicated as a result of the occurrence of biennial spawning (Chapman, 1980; Sterk and Redant, 1989 [cited by Sardà 1991]; Sardà, 1991), although it

appears that the factors that cause a proportion of the population to spawn biennially have not been specifically identified.

A mature male will only become sexually responsive to a female that has recently moulted, and only for the short period that her exoskeleton is soft. The male elicits a characteristic behavioural activity prior to copulation, which is thought to be pheromone induced, since a male will still respond in this manner when placed in a tank from which a recently postmoult female had been removed (Farmer, 1974). The majority of females will carry spermatophores during the period prior to oviposition and will remain confined within their burrows once spawning has taken place. This results in a high male/low female proportion obtained in trawl catches (See Farmer, 1975; Chapman, 1980; Orsi-Relini and Relini, 1989; Sardà, 1991). Table 1.1 details the female reproductive cycle, however, it should be noted that the data is generic due to latitudinal variation. For reasons of simplicity the data concerning biennial spawning have not been included in table 1.1.

Nephrops norvegicus may be expected to produce, depending on size and age of the animal, between 250 and 2000 pelagic larvae for each reproductive cohort (Chapman, 1980). There are three zoea stages (Figueiredo and Thomas, 1967) and according to Farmer, (1975), one non-swimming pre-zoea which maintains an embryonic shape. The initial moult of the pre-zoea occurs soon after hatch, giving rise to the first of three free-swimming zoea, each increasing in total body length from 5.5—7mm for the first zoea, 7.5—10mm for the second and finally to 10.5—12mm for the third zoea (see Farmer, 1975). The duration of each larval stage is largely temperature dependent; the first zoea, for example, when maintained at 7—10°C, lasts 14—15 days before the next moult, although at 13—17°C this stage timing is reduced to 5—6 days (Figueiredo and Vilela, 1972). The entire larval development lasts for 2—3 weeks before metamorphosis into a juvenile (see Farmer, 1975). At metamorphosis, juvenile *Nephrops* have a

Table 1.1

The reproductive cycle of the female *Nephrops norvegicus*.

September-October	1st year	Commencement of ovarian maturation *(Farmer, 1974; Farmer, 1975; Orsi-Relini and Relini, 1989; Sardà, 1991)*
May-August	2nd year	Female exuviation, is followed by copulation by a premoult male *(Lüling, 1958; Farmer, 1974; Orsi-Relini and Relini, 1989)*
August-September	2nd year	Fertilisation and spawning *(Orsi-Relini and Relini, 1989; Sardà, 1991)*
September to April/May	2nd year	Incubation of eggs on pleopods, next brood developing in ovary *(Figueiredo and Barraca, 1963; Figueiredo and Thomas, 1967; Farmer, 1975)*
April-June	3rd year	Egg hatch and larval release *(Farmer, 1975; Chapman, 1980; Orsi-Relini and Relini, 1989)*
May-August	3rd year	Female exuviation, is followed by copulation by a premoult male
August-September	3rd year	Fertilisation and spawning (second reproductive cohort)

carapace length of 3—4mm (Farmer, 1975; Figueiredo, 1975) and although most settle to the sea bed at this stage, few are caught in commercial trawls (Chapman, 1980). During the first year, the juveniles may moult up to ten times, to attain a size of up to 15mm carapace length (Farmer, 1975), although most of this time is spent within adult burrows, from which the juveniles rarely emerge (Chapman, 1980). Photographic data (Chapman, 1980) shows a gradual increase of individuals over 13mm carapace length on the sediment surface. In addition, Chapman (1980) describes data that show that stomach contents of predatory fish rarely contain the remains of juvenile *Nephrops*. These data lend support to the theory that juveniles do not emerge from the burrow during the first year of development.

The processes of somatic growth, moulting and reproduction in decapods are closely interlinked (Skinner, 1985a; b). Energy is required to be apportioned between both somatic growth and reproductive processes in order that neither are favoured to the detriment of the other (Sardà, 1991). For example, *Nephrops norvegicus* display three moult periods: the first "fixed" moulting period from December to April, observed in both sexes, which is followed by an intermoult period of 3—4 months. The second moult period, for the smaller size class animals, occurs during June/July. The third occurs in September/October which affects most of the males and juvenile females. The coordination with timing of reproduction has been well documented (Karlovac, 1953; Figueiredo and Barraca, 1963; Figueiredo and Thomas, 1967; Farmer, 1974; Sardà, 1991). Gonad maturation and oviposition occurs when the ecdysial frequency of the female *Nephrops* is lowest, while eclosion of ova is concurrent with the onset of the "fixed" moult period. Furthermore, copulation will take place during February and April between a female, recently moulted during the "fixed" winter period, and a male that may have moulted in the September/October period (Sardà, 1991). As discussed earlier, there may be a certain amount of latitudinal variation and local

differences in the reproductive periods (Bailey, 1984; Orsi-Relini and Relini, 1989). The differences in depth between populations in the North Atlantic (20—500m) and those in the Mediterranean (300—600m) will create quite distinctly different environments and ultimately will effect both the moult and reproductive cycles and the synchrony that occurs between them (Orsi-Relini and Relini, 1989; Sardà, 1991).

The endocrinology of *Nephrops norvegicus* has not been investigated in any detail. The omission of this area of study is clearly illustrated by the literature concerning moult and moulting cycles discussed above. Ecdysis is expressed only with reference to somatic growth, frequency and actual moult staging, (Figueiredo, 1967; Farmer, 1975; Sardà, 1981) and rarely is any mention made of moulting hormones. In actuality, previous to Farmer's review paper (1975) only tentative investigations of eyestalk neuropeptides had been carried out (Carstam, 1942; Kleinholz *et al*, 1962). However, migration of accessory pigment from the within the eye of *Nephrops* (Shelton and Gaten, 1986) and the partial characterisation of red-pigment-concentrating hormone (RPCH) from the same species (Gaus *et al*, 1990) are two contemporary studies which show a renewed interest in the endocrinology of the species. Leuven *et al*, (1982) discussed crustacean hyperglycaemic hormone (CHH) of a number of species, including *Nephrops*. The consideration of *Nephrops* in this study, demonstrated the effect of eyestalk extract injections and clearly shows species specificity of *Nephrops* to other genera within the Astacidea while additionally displaying a non-specificity to eyestalk extracts from Brachyura.

Recently, problems within the fishing industry have brought *Nephrops norvegicus* to the attention of scientists, other than those interested in its reproductive strategies, moult staging and fishing quotas (Hagerman and Uglow, 1985; Hagerman and Baden, 1988; Hagerman *et al*, 1990; Baden, *et al*, 1990; Spicer *et al*, 1990). These investigations have shown a positive link between the

ecological and the biochemical aspects of the biology of *Nephrops norvegicus*. Spicer *et al* (1990) noted that recently collected animals were unable to survive longer than 18 hours experimental emersion. During this time the animal enhanced normal cellular energy demands by anaerobic metabolism. This connection between fishing and anaerobism has been further investigated in relation to the metabolism of the animal under hypoxic stress in its natural environment (Hagerman and Uglow, 1985; Hagerman and Baden, 1988; Hagerman *et al*, 1990; Baden *et al*, 1990). Commercial catches of *Nephrops* in the Kattegat, a region between the Danish and Swedish coasts, yielded high concentrations of dead animals and this has been linked to periods of severe hypoxia as a result of an increase of eutrophication and pollution in the area. The anaerobic metabolism of the *Nephrops*, both in its natural habitat and under artificially induced hypoxic conditions was investigated, again showing the animals short term adaptation to the altered conditions. These papers will be discussed in more detail at a later stage and will be compared to investigations in this current thesis. (See "Physiological stress imposed on *Nephrops norvegicus* by severe hypoxia", Chapter 5). Apart from these relatively recent papers, the majority of work on *Nephrops* has had an ecological rather than biochemical emphasis.

Detailed work on the endocrinology of other commercially important species, and even crustaceans of a lesser commercial value, is at a very advanced level when compared to that of *N. norvegicus*. For example, the moult cycle and ecdysteroid titres of the lobster, *Homarus americanus*, have been elucidated (Aiken, 1980; Chang, 1984, 1985; O'Connor, 1985; Skinner 1985a; b) as has that of the fiddler crab, *Uca pugilator* (Hopkins, 1983, 1986). A moult inhibiting hormone from the shore crab, *Carcinus maenus* has been purified by Webster and Keller (1986) with an amino acid sequence elucidated for the same species (Webster, 1991). Gonad Inhibiting Hormone, GIH (until recently named vitellogenesis inhibiting hormone, VIH, see later) has been characterised and

sequenced by Soyez *et al*, (1991) from the lobster, *Homarus americanus* and by Huberman *et al* (1992) from the crayfish, *Procambarus bouvieri*. Investigations into the neuropeptide crustacean hyperglycaemic hormone (CHH) are extensive. The sequence was elucidated by Kegel *et al*, (1989) from the shore crab, *Carcinus maenus*. Since this study, homologous peptides from the crayfish, *Orconectes limosus* (Kegel *et al*, 1991) and the lobster, *Homarus americanus* (Chang *et al*, 1990) have been sequenced, the latter being described as a moult inhibiting peptide which also possesses hyperglycaemic activity. Sequencing of CHH from the terrestrial isopod, *Armadillidium vulgare*, has shown a very similar sequence to that of the decapod peptides (Huberman *et al*, 1993; Martin *et al*, 1992; Martin *et al*, 1993).

Reasons for the disparity of information between *Nephrops* and the afore-mentioned species is peculiar, although it may be explained by the technical difficulties of working with *Nephrops* as a laboratory animal. The species is very delicate; trawls are notorious for fatally damaging the animal (Simpson and Symonds, 1968; Symonds and Simpson, 1971), while creels yield such low results that they are not a profitable means of capture to the researcher. Mortality in aquaria is very high; an animal with both chelipeds attached has only a 69% chance of survival, while an individual with one or no chelipeds have a 50% and 22% chance of survival respectively (Symonds and Simpson, 1971). Furthermore, mortality is apparently increased due to eye damage caused by high surface light intensities (Loew, 1974).

1.2 An introduction to crustacean endocrinology.

The role of the endocrine system in insects has been intensely investigated over a number of years. In contrast, the interest in the endocrinology of crustaceans is relatively recent and although great advances in this area have

created a better understanding of the crustacean system, there is much that remains to be investigated. The principal endocrine structures of crustaceans are the eyestalks and their associated structures, in particular the X-organs sinus gland complex. The moult cycle and somatic growth, reproduction, carbohydrate homeostasis, chromatic variation and osmotic regulation are all under the direct control of this system. Other endocrine structures within the crustaceans mediate numerous metabolic processes and all to a greater or lesser degree are considered to be under the influence of the eyestalk and its secretions. These include the Y-organ, which controls the release of ecdysteroids, the pericardial organs (PO's) whose secretions effect cardioexcitatory activity and the post-commissural organs, which may be involved in the dispersal and concentration of integumental pigments and like the (PO's) may have a neuromodulatory role. Each of these endocrine structures will be discussed in more detail later in this chapter (Beltz, 1988 for review). The principal differences between (neuro)endocrine and the neuronal control of the transmission and release of bioactive factors is summarised in figure 1.1.

Hormones synthesised, often as the inactive precursor, on the rough endoplasmic reticulum (RER), are condensed into vacuoles in the Golgi complex and are transported axonally as neurosecretory granules. Terminally, the neurosecretory axons branch and proliferate in contact with the blood system to form a neurohaemal organ (Carlisle and Knowles, 1953). Electrical stimulation of the cell body or neuropile generates an increased permeability to sodium and calcium ions (Na^+ and Ca^{2+}) and results in an action potential, which relying on Na^+ entry, is propagated along the axon. The resulting depolarisation of the nerve terminal promotes Ca^{2+} entry, which in turn induces hormone release directly into the blood system (for review see, Bern and Hagadorn, 1965; Maddrell and Nordmann, 1979; Scharrer, 1990).

Figure 1.1

The representation of the main differences between a typical neurosecretory cell (left) and a non-neurosecretory cell (right) – in this case a cholinergic neurone. The neurosecretory cell has a large number of nerve endings, each releasing into circulation, hormone (H), which is synthesised in the cell body and transported along the axon endings. The cholinergic neurone has many fewer endings which release transmitter (acetylcholine) into the limited space of the synapse; the transmitter is assembled in the nerve endings themselves in order that axonal transport is not so important. (This diagram is a slightly modified version from Maddrell, S.H.P., and Nordmann, J.J., (1979) The phenomenon of neurosecretion, in Tertiary level biology. Neurosecretion, Blackie and Son Limited, ch. 1 pp 7-8. (Reproduced with permission).

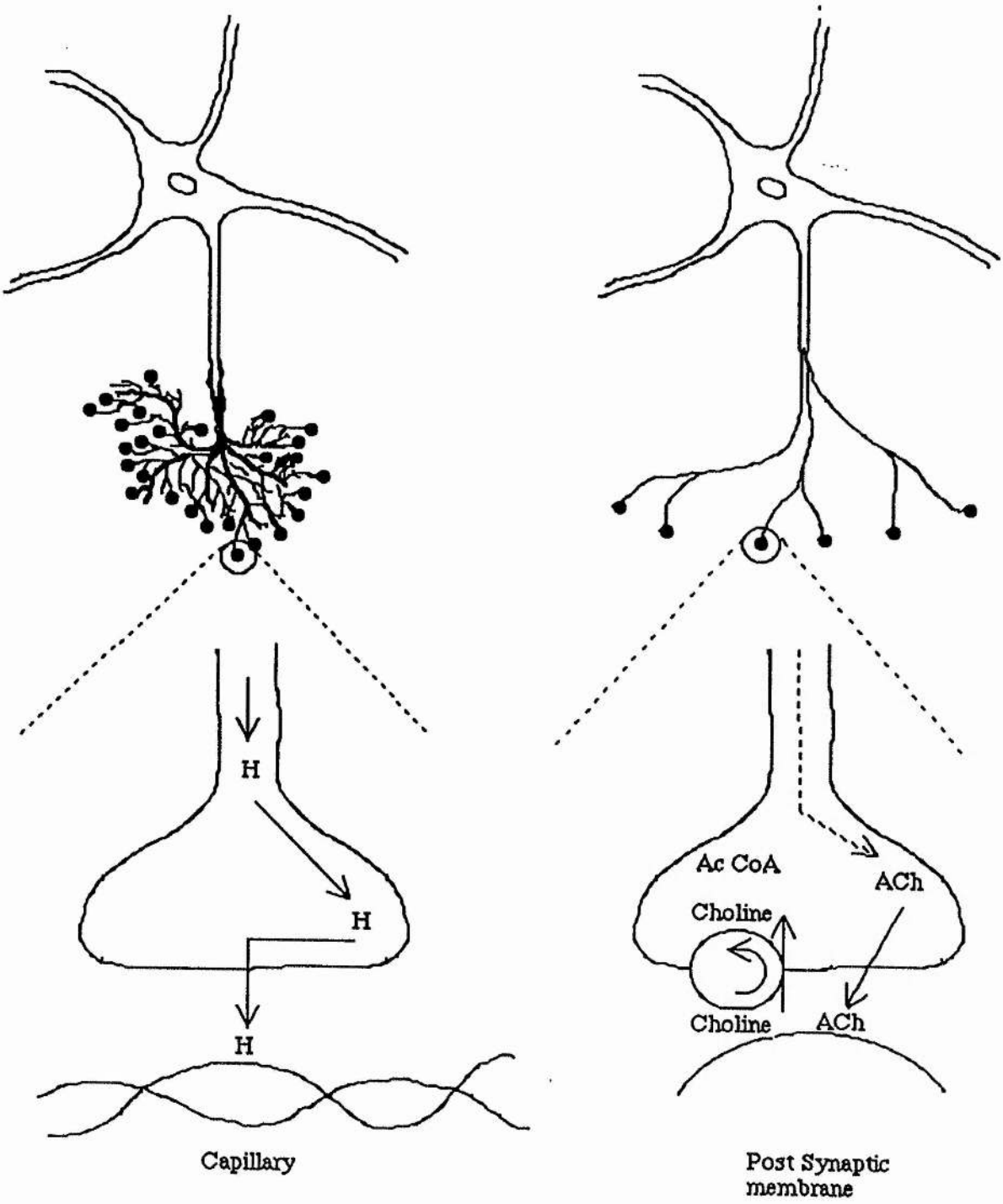

H
H
H
Capillary
Ac CoA
ACh
Choline
Choline
ACh
Post Synaptic membrane

Table 1.2

Examples of endocrine mediated phenomena in the Crustacea: short, medium and long term regulation.

Short term control	Chromatophore adaptation	*Kleinholz, 1976; Quackenbush & Fingerman, 1984.*
	Cardiac regulation	*Maynard & Welsh, 1959; Florey & Rathmayer, 1978; Stangier et al, 1987.*
	Locomotion	*Kravitz et al, 1980; 1985.*
	Retinal pigmentation	*Fingerman & Mobberly, 1960; Fernlund, 1976.*
Medium term control	Water and ion balance	*Kamemoto & Tullis, 1972; Mantel, 1985.*
	Carbohydrate metabolism	*Keller et al, 1985; Keller & Sedlmeier, 1988.*
	Respiration	*Keller & Orth, 1990.*
Long term control	Growth, ecdysis and regeneration	*Skinner, 1985; Chang & O'Connor; 1988.*
	Reproduction	*Payen, 1986; Charniaux-Cotton & Payen, 1988.*
	Endogenous rhythms*	*Hamann, 1974; Aréchiga & Huberman, 1980; Aréchiga et al, 1985, Kallen et al, 1990; Keller and Orth, 1990.*

* The input of external factors into the eyestalk system, ultimately controls endogenous rhythms within the crustacean. Inclusion of endogenous rhythms as a long term controlling endocrine mediated phenomenon refers to the release and maintenance of circulating peptide hormones and not necessarily to the external input.

The neuroendocrine system of invertebrates is well suited to the study of neurobiological mechanisms of adaptive physiology. The crustacean system displays major adaptive variations which are manifest as short, medium or long term regulation (Tensen, 1991). Short term regulation should be understood as effects that may only last for minutes, medium term may have a duration of hours, while long term effects would be expected to occur for days, weeks or even months. Table 1.2 describes a few examples of each of these regulation types that are exhibited in the Crustacea (for reviews see Cooke and Sullivan, 1982, Kleinholz, 1985; Quackenbush, 1986; Webster and Keller, 1987, Beltz, 1988; Keller, 1992).

Neurosecretory cells are mainly grouped within the eyestalk and the cerebral ganglia, although sites of neurosecretion are also present within the sub-oesophageal thoracic and abdominal ganglia. There are three neuroendocrine regions within the crustacean that can be classed as neurohaemal organs, these are the pericardial organs, the postcommissural organs and the sinus glands (Cooke and Sullivan, 1982; Andrew, 1983; Chaigneau, 1983). It was in reference to the former of these that Carlisle and Knowles (1953) defined the term "neurohaemal" a specialised arrangement in which nerve terminals form intimate contact with the circulatory system. These neurohaemal structures will now be discussed, giving a review of the structure and neuroendocrine function of each. The main focus of this thesis involves the investigation of sinus gland neuropeptides, and this will therefore be discussed in more detail, following the consideration of other neuroendocrine structures in the crustacean.

1.2.1 The pericardial organs.

The crustacean pericardial organs (PO's) lie in the venous cavity surrounding the crustacean heart and, although there is anatomical variation within

the Malacostraca (stomatopods and decapods), the majority release hormones into the circulatory system. These include biogenic amines and cardioexcitatory peptides (Cooke and Sullivan, 1982). The axons of the PO's originate in the thoracic ganglia and peripheral tissues (Alexandrowicz, 1953a; b) and are the site for the release of biogenic amines, such as octopamine, dopamine and serotonin (5-HT) (Florey & Rathmayer, 1978), into the haemolymph, although other sites for their release are possible (for review see Cooke and Sullivan, 1982; Quackenbush, 1986; Beltz, 1988; Keller, 1992). Recently, serotonin and dopamine have been identified as possible regulators of CHH synthesis and release, mediated by synaptic input of CHH axon ramifications in the medulla terminalis (Gorgels–Kallen, 1985; Van Herp and Kallen, 1991). It was thought for some time that serotonin was the major PO factor that effected the heart (Florey & Florey , 1954). It was shown however, that the circulating levels were too low to account for the cardioexcitatory activity observed and in addition, the pattern of response that cardiac muscle showed to PO extract was different to that displayed by serotonin (Maynard & Welsh, 1959). The pentapeptide proctolin, was therefore proposed, as the main cardioexcitatory peptide associated with the PO's and was first isolated from the cockroach hindgut (Brown and Starratt, 1975; Starratt and Brown, 1975; Sullivan, 1979). Studies on proctolin have displayed a wide stimulatory role on different muscles and neurones, and its isolation, immunochemical localisation and chemical characterisation from the PO of *Homarus* and *Carcinus* has been demonstrated (Schwartz *et al*, 1984, Stangier *et al*, 1986). The presence of proctolin in the PO's of Crustacea suggest that the peptide has a neurohormonal role in the group, although circulating levels have not to date been detected. The perikarya that give rise to the neurohaemal tissue have been mapped immunocytochemically in the thoracic ganglion-PO's system of *Carcinus* (Dircksen *et al*, 1987) and proctolin immunopositivity has been detected throughout the nervous system of *Homarus* and *Procambarus* (Siwicki and Bishop, 1986). It appears that the release of proctolin can be local, or from sites such as the

PO's, directly into the haemolymph. The release of the peptide from the PO's points to their role as neurohormones, although the peptides distribution in the nervous system suggests that it may be acting locally as a transmitter or neuromodulator (Orchard *et al*, 1989; Keller, 1992). This neuromodulator role of proctolin has been demonstrated by the stimulation of pleopod activity in crayfish (Mulloney *et al*, 1987) and has been shown to initiate and modulate gastric mill chewing (Heinzel, 1988).

Crustacean Cardioactive Peptide (CCAP), a nonapeptide which, as its name suggests, has cardioactive properties, was first isolated and sequenced from the PO's of *Carcinus maenas* (Stangier *et al*, 1987) and has since been identified in *Homarus* and *Orconectes* (for review see Keller, 1992; Stangier, 1991; Stangier and Keller, 1990). Radioimmunoassay (RIA) has identified the PO's as the major site of CCAP activity, although CCAP-immunopositive neurones have been shown to occur throughout the nervous system of *Carcinus* and *Orconectes* (Dircksen and Keller, 1988; Trube *et al*, 1991) in particular, the 6th abdominal ganglion in *Homarus* and *Orconectes* (Stangier *et al*, 1988; for review see Keller, 1992). There have been only limited investigations into the physiological role of CCAP. The excitatory activity of the peptide on the heart and hindgut of *Orconectes* has been demonstrated (Stangier and Keller, 1990), although the effects on the heart rate of *Cancer borealis* and *Homarus gammarus* were less dramatic (Stangier, 1991). Recent investigations indicated the presence of CCAP throughout the nervous system of *Locusta* (Dircksen *et al*, 1990) and the contractile properties of the peptide on the hindgut and oviduct have been demonstrated in the same species (Stangier *et al*, 1989).

The PO's may, in addition, act as a release site for CHH putatively synthesised in the thoracic ganglia. RIA and anti-*Carcinus* CHH serum were used to demonstrate the presence of CHH in these tissues (Keller *et al*, 1985). Molecular expression of CHH has been demonstrated in the cerebral, thoracic and

abdominal ganglia in *Orconectes* and *Homarus* (Tensen, 1991; Tensen *et al*, 1991a). As previously discussed, the PO's act as a neurohaemal organ with the neurosecretory cells originating in the thoracic ganglia thus providing supporting evidence of the organ's secretagogue properties. This may in turn suggest why destalked animals have been shown to still have measurable levels of CHH in the haemolymph (Keller and Orth, 1990).

1.2.2 The Post-commissural organs.

Post-commissural organs, PCO's, have the morphological features typical of crustacean neurohaemal organs. They have been described in the natantians, *Penaeus brasiliensis* and *Palaemon serratus*, and in the stomatopod, *Squilla mantis* (for review see Carlisle and Knowles, 1959). They are connected to the circumoesophageal commissures (COC) and are known to contain factors causing pigment contraction in white chromatophores (Carlisle and Knowles, 1959). The COC of *Palaemonetes vulgaris* are reported to contain other chromatophoric factors with red pigment dispersing activity (Brown *et al*, 1952). In addition, the PCO's of *Palaemon serratus* have been demonstrated to show gastrin/cholecystokinin (G/CCK) radioimmunoreactive peptides (Dircksen and Van Wormhoudt, in prep see conference abstract). Recent work by Dircksen (1992), displays neurones immunocytochemically specific to antisera raised against red pigment concentrating hormone (RPCH), pigment dispersing hormone (PDH) and G/CCK-like peptides. Two RPCH immunoreactive (IR) neurones from each eyestalk and two G/CCK-IR from the brain terminate in the PCO's, although IR fibres from the latter descend further to the muscles of the stomach. Neurosecretory granules have been identified by ultrastructural analysis of the RPCH-IR PCO terminals (Dircksen, 1992). Injections of PCO extracts cause the complete contraction of red chromatophores for up to three hours (Dircksen,

1992), however, RPCH has been shown to have a neuromodulation effect on the pyloric rhythm in *Cancer borealis* (Nusbaum and Marder, 1988).

1.2.3 The Sinus Glands.

The sinus gland system is the major system of neuroendocrine regulation of crustaceans. Of all the crustacean neurohaemal structures, the sinus gland, which is located in the eyestalks, has been the most fully investigated in terms of its ultrastructure, physiology and its peptide chemistry. This intense investigation is partly due to the size and accessibility both of the secretory terminals and their cell bodies. Peptide hormones released by the sinus glands into the circulation, act on both target tissue and other endocrine structures, and are involved in the processes of moulting, growth, sexual maturation and the regulation of metabolism. In addition, hormones that enable adaptation by the animal to altered environmental conditions are also stored and secreted from the sinus glands. The regulation of haemolymph glucose titres, osmotic and ionic concentration, the ability to control chromatic variation and the maintenance of endogenous circadian rhythms, are all homeostatically maintained by the sinus gland peptides.

1.2.3.1 Historical.

The first studies of the eyestalk optic ganglia and the neuroendocrine structures located therein were carried out by Bellonci in 1882 (see Tensen, 1991). His identification of a distinct structure of nervous tissue led to the naming of the X-organ, so called because of its unknown function. Hanström first described the sinus gland in 1931 and due to its proximity to blood vessels, suggested a possible endocrine function. The identification of the four optic ganglia was also made by Hanström; the lamina ganglionaris, medulla externa, medulla interna and the

medulla terminalis and due to the position of the of the cluster of cell somata in close proximity to this latter ganglion, named them Medulla Terminalis Ganglionic X-organ. He also identified a nerve connection between the MTGXO and the sinus gland. The MTGXO is the main source of the axons forming the sinus glands and is consistently present thoughout the Crustacea, however, ganglionic X-organs exist also in the medulla interna and the medulla externa (Knowles and Carlisle, 1956)

In its simplest form, identified by Hanström (1948) in mysids and euphausiids, the sinus gland consists of a thickened disc on the neurilemma of the Medulla Terminalis, bordering a blood sinus, although in the higher decapods the structure is more complex, with a palisade cup-shape surrounding a highly vascularised area. Initially, it was thought that the sinus gland was a site of hormone production, and in many respects the early experiments of eyestalk ablation supported this view. Improved surgical techniques, however, found that sinus gland expiration alone did not duplicate many of the physiological results found in the more drastic ablatory experiments (Frost *et al*, 1951; Bliss, 1953; Passano, 1953). Bliss and Welsh (1952) concluded that the sinus gland was an aggregation of axonal endings from neurosecretory cells of the eyestalk and elsewhere. The use of electron microscopy identified axons filled with neurosecretory granules within the sinus glands (Hodge and Chapman, 1958; Knowles, 1959).

Once it had been demonstrated that the X-organs were the site of synthesis of neurosecretory material and that the sinus gland was acting purely as a neurohaemal organ (Bliss, 1951; Enami, 1951; Passano, 1951), neurohormonal roles were attributed to a number of formerly reported factors pertaining to the eyestalks, the site of synthesis being the X-organs and the site of storage and release, the sinus glands. The innervation of neurosecretory neurones from the X-organ into the sinus gland (Andrew and Saleuddin, 1978; Jaros, 1978; 1979) and

Figure 1.2

Schematic diagram showing the typical internal structures of the crustacean eyestalk. Abbreviations: *LG* lamina ganglionaris; *ME* medulla externa; *MI* medulla interna; *MT* medulla terminalis; *MTGX* medulla terminalis ganglionic X–organ; *NO* nervus opticus; *O* ommatidia; *SG* sinus gland; *T* tract. (Reproduced from Gorgels–Kallen, 1985 with permission).

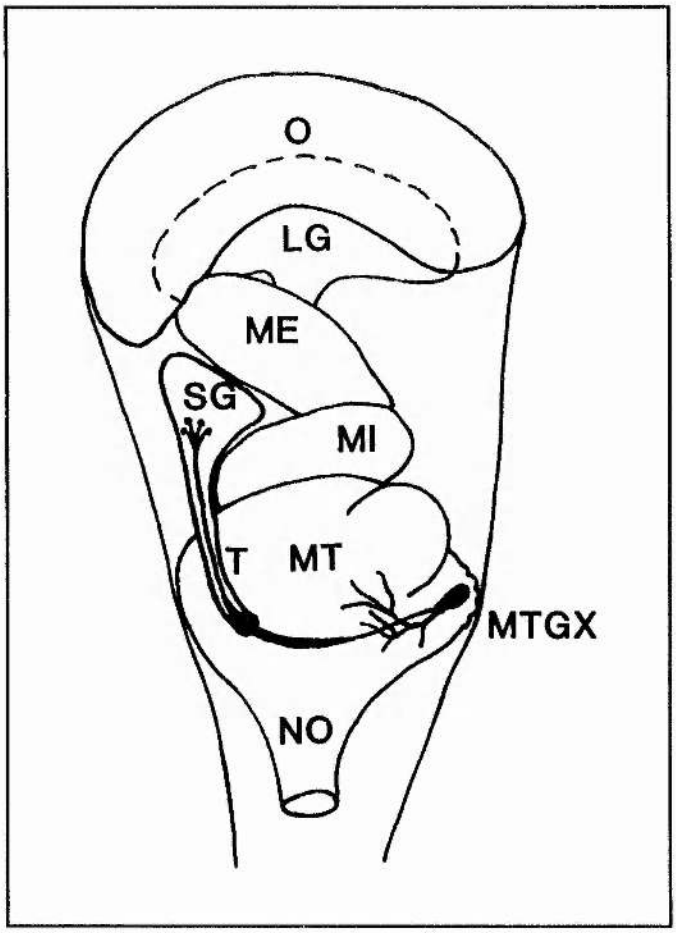
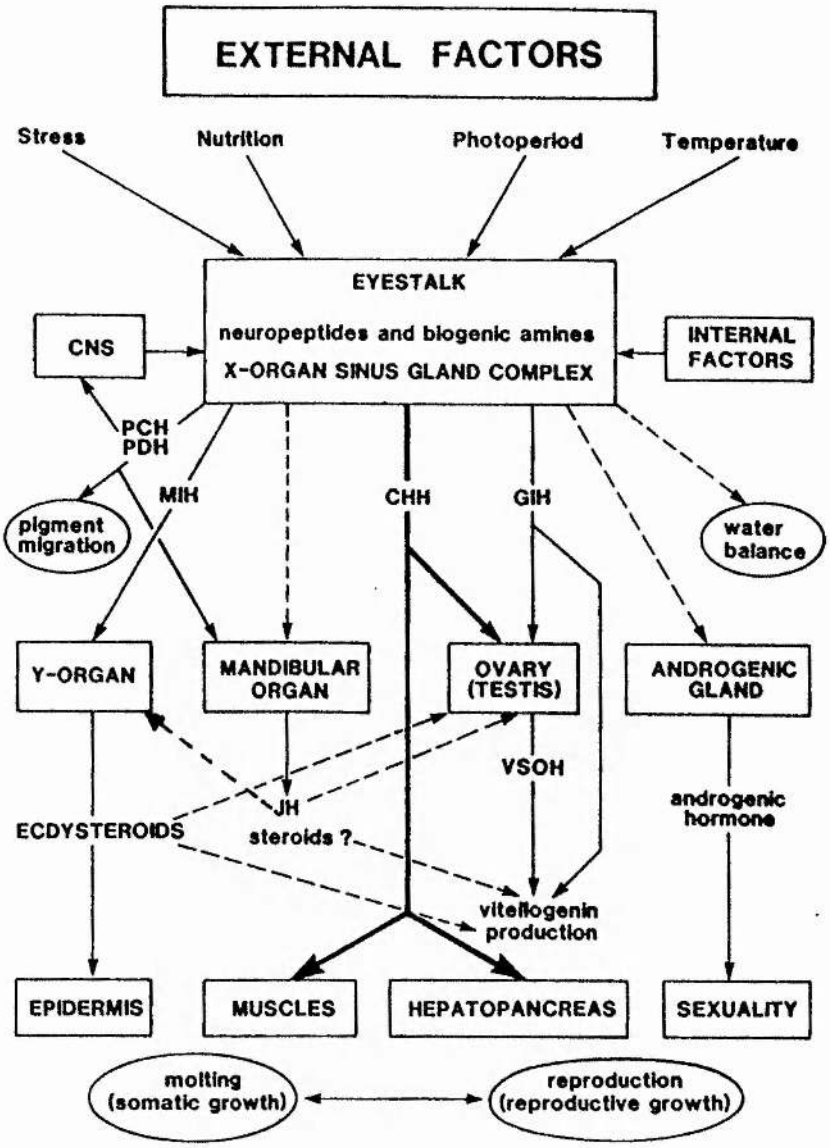

O
LG
ME
SG
MI
T
MT
MTGX
NO

Figure 1.3

Diagram illustrating the integratory role of the eyestalk neuroendocrine system in the (neuro)hormonal control of the physiology of the decapod crustaceans. Abbreviations: *CHH*: crustacean hyperglycaemic hormone; *CNS*: central nervous system; *GIH*: gonad inhibiting hormone (or *VIH*: vitellogenesis inhibiting hormone); *JH*: juvenoid hormones; *MIH*: moult inhibiting hormone; *PCH*: pigment concentrating hormones; *PDH*: pigment dispersing hormones; *VSOH*: vitellogenin stimulating ovarian hormones. (Dashed lines indicate putative control pathways). Reproduced from Tensen (1991) with permission.


EXTERNAL FACTORS
Stress
Nutrition
Photoperiod
Temperature
EYESTALK
neuropeptides and biogenic amines
X-ORGAN SINUS GLAND COMPLEX
CNS
INTERNAL FACTORS
PCH
PDH
MIH
CHH
GIH
pigment migration
water balance
Y-ORGAN
MANDIBULAR ORGAN
OVARY (TESTIS)
ANDROGENIC GLAND
VSOH
androgenic hormone
JH
ECDYSTEROIDS
steroids ?
vitellogenin production
EPIDERMIS
MUSCLES
HEPATOPANCREAS
SEXUALITY
molting (somatic growth)
reproduction (reproductive growth)

immunocytochemical investigations of the MTGXO-sinus gland (MTGXO-SG), where antisera were used to identify specific peptidergic cells and neurones in the optic ganglia (Van Deijnen *et al*, 1985), advanced the understanding of the eyestalk structure and the neuropeptides occurring therein. Figure 1.2 shows a schematic representation of a crustacean eyestalk sectioned longitudinally in order that the optic ganglia and the positioning of the X-organ sinus gland complex can be visualised. Photographs of whole mount preparations and stained sections showing these structures in *Homarus gammarus* and *Nephrops norvegicus* can be seen in Chapter 3.

The homeostatic regulation of biological and physiological processes of the Crustacea by the production and secretion of neuroendocrine peptides in response to external and internal stimuli is therefore the primary role of the MTGXO-SG complex. The multi-functional role that the eyestalk and associated endocrine products has on the biology of the animal and the effects of external, environmental and internal stimuli have on the crustacean is summarised in figure 1.3. Eyestalk ablation has an obvious and drastic effect on the animal's biology by creating an artificial physiological deficiency. Whilst these experiments are severe in their nature, it is likely that they do have a degree of validity. The existence of many of the putative neuroendocrine signals that were investigated and identified by ablatory experiments have, therefore, been indicated with dashed lines (Tensen, 1991).

The remainder of this introduction will deal with only those neuroendocrine peptides that have been experimentally identified and confirmed as eyestalk factors, in particular, those which are synthesised and liberated from the MTGXO-SG complex. The crustacean hyperglycaemic hormone (CHH) will be discussed in greater detail than other eyestalk neuropeptides, not only because it has been historically the most investigated, but also because the aims of this thesis are to

investigate CHH and its physiological significance. This chapter will therefore, provide a recent review of the literature concerning this particular peptide.

1.3 Eyestalk neuropeptides.

1.3.1 Chromatophorotropins.

The chromatophorotropins are peptides classically termed as pigmentary effector hormones, the role of which is to adjust the colour or hue of an animal by the diffusion or concentration of pigment in the chromatophores of the epidermis as well as those situated in the ommatidia of the eye or in some internal organs. Details of the work relating to chromatophorotropins has been reviewed by Rao (1985).

1.3.1.1 Red pigment concentrating hormone.

The first demonstrations that chromatophore control in the shrimp, *Palaemonetes*, were caused by a substance originating in the nervous system were made by Koller (1928), Perkins (1928) and Brown (1933). This substance was red pigment concentrating hormone (RPCH), an octapeptide whose amino acid sequence and chemical synthesis was elucidated from the prawn, *Pandalus borealis* (Fernlund and Josefsson, 1972; Fernlund, 1974a;b). This was the first crustacean neuropeptide whose structure was fully elucidated. RPCH has since been elucidated from three further decapod species (Gaus *et al*, 1990), and its structure is related to the insect peptide adipokinetic hormone (AKH) family. These insect peptides regulate various physiological processes including hyperglycaemia, lipid mobilisation and cardioactivation (for review see Wheeler *et al*, 1988). There are approximately twelve AKH peptides that have had their structure elucidated (for review see Holman *et al*, 1988). There is, however, a surprisingly high degree of sequential variation between the insect species. In

Crustacea this does not appear to be the case as RPCH shows a high degree of conformity of the sequence between investigated species (Gaus *et al*, 1990). Another interesting point of variation between RPCH and AKH is that the latter has two forms (three in *Locusta*) while the former has only the one. A restrictive ligand-receptor relationship may have hypothetically imposed evolutionary constraints on the sequence of RPCH (Gaus *et al*, 1990; for review see Keller, 1992), although in the insect system a gene duplication may have evolved in order that functional modifications of the AKH-like peptides could arise. The structural significance and difference between these two peptide groups and other similar peptides is reviewed by Gaus *et al* (1990), Gäde (1991) and Keller (1992), and will, therefore, not be discussed in detail here.

The functions of RPCH may extend considerably beyond the well established hormone action on the chromatophores. Investigations have shown a possible neurotransmitter or neuromodulator function for the peptide, in the light of immunocytochemical studies on *Carcinus* and *Orconectes* that have identified not only eyestalk neurones projecting into the sinus gland that have neurosecretory properties, but additional neurones with no connections to neurohaemal regions (Mangerich *et al*, 1986).

The demonstration of a neurological role for RPCH acting as a neuromodulator on the pyloric rhythm in the crab, *Cancer borealis* and the presence of RPCH-immunopositive neuronal innervation to the stomatogastric ganglion, supports this multi-functional theory for RPCH (Nusbaum and Marder, 1988). Prestwich *et al* (1990) reported the characterisation of a neuropeptide binding protein for RPCH in neural tissues which suggests a role for the peptide as a neurotransmitter in the CNS. A more recent study gives evidence that RPCH acts as an excitatory modulator on the pleopod of *Pacifastacus leniusculus* (Sherff and Mulloney, 1991). Isolated preparations of nerve cord display rhythmic bursts of activity when perfused with RPCH while RPCH-immunopositive perikarya and

fibres in the abdominal ganglia authenticate these findings. Finally, the stimulatory release of methyl farnesoate by RPCH from the mandibular organs of crustaceans , suggests a reproductive role for the chromatophorotropin (Laufer *et al*, 1987).

1.3.1.2 Pigment-dispersing hormones.

Pigment-dispersing hormones (PDH's) were originally termed light-adapting distal retinal pigment hormone (DRPH). This is an octadecapeptide that was first isolated from the eyestalks of *Pandalus borealis* (Fernlund, 1976) and was shown to control both retinal pigment migration and the dispersal of chromatophore pigments (for review see Rao, 1985). The isolation of four different PDH's from five crustaceans and two from insect species, reveal a peptide family (Rao and Riehm, 1989) and have shown that the sequence shows some heterogeneity between species, differing therefore to the highly conserved RPCH. A conserved region of the PDH, amino acids 5—10, is thought to be the message region for the peptide (Rao and Riehm, 1989). The identification of PDH in insects, using the crustacean chromatophores as a bioassay (Rao and Riehm, 1989), indicates the peptide appears not to act in a classical hormonal manner within this group.

Immunocytochemical investigations have identified PDH containing neurones in the MTGXO and the sinus gland. In addition, as with RPCH, a non-hormonal neuromodulator role for PDH was suggested, as immunocytochemistry identified neurones of both secretory and non-secretory properties in other areas of the eyestalk and central nervous system (Mangerich *et al*, 1987; Mangerich and Keller, 1988). Although PDH immunopositive staining of neural innervation into the stomatogastric ganglion is similar to other known neuromodulators, it should be noted that there appears to be a degree of phylogenetic variability in the

antibody staining. *Cancer borealis* and *Cancer antennarius* are a case in point; each displaying differences in the localisation of PDH (Mortin and Marder, 1991). Finally, it is known that PDH inhibits the release of MF (Laufer *et al,* 1987) and it is possible that it is an antagonist to RPCH.

Peptide families may contain a large number of related peptides that may have slightly differing amino acid sequences (Thorndyke, 1988). Antibodies that are raised against one peptide within a family may also detect for another related peptide. Conversely, an antibody that does not detect a specific peptide in a tissue or neurone may still detect a related peptide, possibly with a similar biological function. The comparison analysis of peptides by the use of immunolabelling is complicated by this, although phylogenetic differences in immunocytochemistry indicate a change in the peptide content which may be unconnected with biological function (Katz and Tazaki, 1992).

1.3.2 Gonadotropins.

As mentioned above (page 11) there is a relationship between somatic and gonadal growth in decapod crustaceans. Reproduction takes place during the intermoult period, and, although in synchrony with the moult cycle, there may be several moult cycles during a single reproductive period (Adiyodi, 1985; Charniaux-Cotton, 1985; Charniaux-Cotton, and Payen, 1988; Meusy and Payen, 1988).

Vitellogenesis is the process by which yolk is deposited within the developing oocyte. There is no clear stage at which this process starts and so the term early vitellogenic phase or primary vitellogenesis is used to refer to the period preparatory to vitellogenesis (Adiyodi and Subramoniam, 1983). Vitellogenesis can be divided into two phases. Primary vitellogenesis is a period of slow oocyte

growth while secondary vitellogenesis, in contrast, is a period of rapid ovarian growth immediately preceding oviposition, during which, there are large increases in gonad weight, with yolk coming both from the gonad itself (endogenous source) and from the hepatopancreas (exogenous source) (Charniax-Cotton, 1985; Blanchet-Tournier, 1982). Female reproduction is regulated mainly at the time of secondary vitellogenesis.

Reproduction and its regulation in the Decapoda is under the inhibitory control of an eyestalk neuropeptide. Panouse (1943) demonstrated that eyestalk ablation during genital quiescence removed this inhibition, stimulated the rapid development of the ovary in the shrimp *Leander serratus*, and resulted in precocious egg deposition. This effect was not observed in juvenile females (Panouse, 1946). The presence, therefore, of an ovarian inhibiting hormone in the eyestalk had been demonstrated. This hormone was to be known as gonad inhibiting hormone (GIH) as it retards gonad maturation and secondary vitellogenesis. The MTGXO was later identified as the site of neurohormonal synthesis and release (for review see Kleinholz and Keller, 1979). The role of GIH has been confirmed in a number of decapod species (for reviews see Charniaux-Cotton and Kleinholz, 1964; Adiyodi and Adiyodi, 1970; Demeusy, 1970; Adiyodi and Subramoniam, 1983; Charniaux-Cotton, 1985; Charniaux and Payen, 1988). Until recently, however, the term vitellogenesis inhibiting hormone or VIH was considered to be more accurate due to the peptides inhibitory activity on the secondary vitellogenic phase. To complicate the nomenclature of this peptide still further, Van Herp *et al* (1992 conference abstract) noted the presence of GIH/VIH in the X-OSG complex of the male lobster, and suggests the peptides role in male reproductive strategies. The name GIH (gonad inhibiting hormone) instead of VIH (vitellogenesis inhibiting hormone) is suggested by the authors as this encompasses the general function of the neuropeptide. As this is likely to be a particular area of debate in the future and as the presence of GIH has not been demonstrated as an

active peptide within the male reproductive system, GIH will be used to describe this hormone throughout this thesis.

By the use of a heterologous *in vivo* bioassay, which measured the retardation of oocyte growth in the prawn *Palaemonetes varians*, the purification and characterisation of sinus gland peptides representing GIH was possible from *Homarus americanus* (Soyez *et al*, 1987; Tensen *et al*, 1989; Van Deijnen, 1986). Characterisation of GIH from the crayfish, *Procambarus bouvieri* by Aguilar *et al* (1992) indicated a single peptide of 8388Da. However, Soyez *et al* (1990), isolated two related peptides of approximately 8kDa from *Homarus americanus* and a complete amino acid sequence for these neuropeptides with a Fast Atom Bombardment Mass Spectrometry (FAB/MS) determined molecular weight of 9135Da, has now been established (Soyez *et al*, 1991). Each isoform is a neutral (PI 6.8) 77 amino acid long peptide, with a free N–terminus, six cysteine residues and is devoid of CHH activity (Tensen, 1991). The structural significance of these isoforms of GIH will be discussed in section 1.3.4.2 under the structural aspects of CHH. Alignment of sequences of *Homarus* GIH and *Carcinus* MIH show a closer relationship between these two peptides than that with CHH from the same species and it is clear that these are two separate types of peptide that have diverged considerably from CHH (Keller, 1992). There is no homology between the sequence of GIH or indeed CHH and MIH with any other known peptide, thus suggesting that this group is unique to the Crustacea.

Polyclonal mouse antisera raised against high performance liquid chromatography (HPLC) purified GIH have been tested against extracts of sinus glands from several crustacean species, including sinus gland peptides from *Homarus americanus*. A preliminary immunocytochemical detection of GIH terminals in the sinus glands of *Homarus americanus* and the wood louse, *Porcellio dilatatus* was demonstrated (Meusy *et al*, 1987). Using both GIH and CHH antibodies, partial co-localisation of both peptides with CHH perikarya in the

MTGXO was identified, resulting in the suggestions that both GIH and CHH are synthesised in the same cells, that they may be encoded by the same RNA transcript or that both may originate from the same precursor. This last hypothesis had been supported by similar reports (Stuenkel, 1986; Eipper and Mains, 1988; Thomas *et al*, 1988). However, recent work by Van Herp *et al*, (1992) demonstrates the detection of the *Homarus* CHH and GIH mRNAs in combination with immunocytochemical techniques, demonstrating a degree of cellular colocalisation for both peptides within the eyestalk. The suggestion that both peptides may be synthesised within one cell group of the X-organ may be premature due to the possibility of three cell groups; one for each peptide and one with the capability of dual synthesis. As the expression appears not to be simultaneous, it is suggested that there is no common precursor and that both peptides originate from different primary transcripts.

Finally, initial studies of the mode of action of GIH suggests the inhibition of receptor mediated endocytotic uptake of yolk by oocytes *in vitro* (Jugan and Soyez, 1985; Jugan and Van Herp, 1989).

1.3.3 Moult-inhibiting hormone.

Crustacea generally undergo incremental growth by a succession of moults, although moulting may take place without any increase in size, or indeed, with an actual decrease. At each normal moult, the old shell is cast and the animal swells by the absorption of water; often by actual drinking, while the epidermis then hardens as the new shell, now slightly larger. This increase in size at moulting is merely an increase in the wet weight of the body. Throughout the rest of the intermoult period, before the next moult, the water is gradually replaced by new tissue, either by swelling of old cells or the formation of new ones; the amount of total protein in the body increases, while that of water decreases. Essentially, all

the tissues will be affected in some way by the moulting process, and it is this pervasiveness of the moult cycle that led to the proposition that the whole cycle is controlled essentially by two hormones; 20 OH-ecdysone and moult-inhibiting hormone (MIH).

The moult cycle is controlled by the release and maintenance of ecdysteroids from the Y-organs affecting many target organs (for review see, Skinner, 1985a; b; Chang and O'Connor, 1988; Watson *et al*, 1989). Zeleny (1905) first demonstrated that eyestalk ablation results in a significant acceleration of the moult cycle, the effect being to trigger premoult. Based upon these observations, it was postulated that a moult inhibiting hormone (MIH) is responsible for supressing the secretion of ecdysteroids from the Y-organ, indeed, eyestalk ablation causes an increase of ecdysteroid synthesis and high titres of ecdysteroids in the haemolymph (Keller and Schmid, 1979; Chang and O'Connor, 1983). Conversely, crude sinus gland injection into eyestalkless animals prevented synthesis and release of ecdysteroids (Keller and O'Connor, 1982; Bruce and Chang, 1984; Snyder and Chang, 1986). The inhibitory effect of purified sinus gland fractions on isolated Y-organ synthesis of ecdysteroids was adopted and resulted in the isolation and identification of MIH from *Carcinus maenas*. In addition, it was demonstrated, that CHH acts in a similar, although less pronounced inhibitory manner on ecdysteroid synthesis (Webster, 1986; Webster and Keller, 1987). The mechanism of this inhibition is unclear, although it is understood to involve cyclic nucleotides. There appears to be differences in the actual nucleotide involved depending on the species. In the crab, *Cancer antennarius*, cAMP was suggested as the second messenger (Mattson and Spaziani, 1985; Mattson and Spaziani, 1986a; b; Wattson *et al*, 1989). The mechanism in *Orconectes limosus* supports cGMP (Sedlmeier and Fenrich, in press). Recent experiments point to a change in the quality of MIH receptors during the moult cycle. Ecdysteroid synthesis could only be inhibited in the stages C and D1, but not in D2 or later

(Sedlmeier, 1992). Receptor binding investigation of MIH from different crustacean species suggest that the region of receptor binding has been highly conserved. This is in contrast to the sequences not involved in binding, which have altered considerably (Webster, 1992). Isolation and characterisation of MIH from the XOSG complex of *Carcinus maenas*, by adopting an inhibitory bioassay on the synthesis of ecdysteroids from the Y-organs, showed that the MIH was in fact related to CHH (Webster and Keller, 1986). This was confirmed by the complete sequencing of *Carcinus* MIH (Webster, 1991). Until recently, MIH has been described as "putative", as its function as a physiological moult regulator has never been demonstrated *in vivo* (Keller, 1992). However, work by Soyez *et al*, (1992) demonstrated an *in vivo* bioassay which lengthened the duration of the moult cycle in juvenile *Penaeus vannamei.*

The structure of *Carcinus* MIH clearly shows that it is a member of the CHH family, the homology of the sequence between the two being 28%. This, however, is not as close as between different CHH's. CHH has 72 residues, while MIH has 78 and in contrast to CHH both termini of the latter are unblocked. Additionally, similarities between the two are only in single or small groups of dispersed amino acids and not as conserved partial sequences that are observed between the different CHH peptides (Keller, 1992). The sequence of *Homarus* GIH (Soyez *et al*, 1991) shows the same percentage of homology with CHH as does MIH, although the homology between GIH and MIH is considerably higher (48%) indicating a closer relationship between these two peptides than with CHH. It has been suggested, therefore, that these similarities indicate a distinct type of peptide that has diverged from CHH. The isolation of a putative MIH has been isolated from the sinus glands of *H. americanus*, (Chang *et al*, 1987) and a sequence of a peptide with *in vivo* MIH activity has been obtained (Chang *et al*, 1990). The HPLC chromatograms of this "MIH", however, display two peptides and its bioassay shows it to be the most potent hyperglycaemic peptide within the

lobster sinus gland. In addition, the sequence is almost identical to the CHH of *H. americanus* as shown by cDNA cloning (Tensen, 1991; Tensen *et al*, 1991b). As only a single step of HPLC purification was adopted by Chang *et al* (1990), it is possible that the MIH effect may be attributed to other coeluting peptide(s) (Tensen, 1991).

Immunocytochemical investigations of *Carcinus* MIH show localised MIH synthesising cells within the X-organ of *Carcinus* and four other species (Dircksen *et al*, 1988) and in the larvae of *Carcinus maenas* (Webster and Dircksen, 1991). In addition, the use of CHH antisera showed two discrete, but intermingled neurosecretory pathways for each of the peptides. Although within the X-organ the cells specific to MIH were intermingled with those specific to CHH and their separate axons, followed the same tract and terminated in a similar pattern within the sinus gland, there is apparently no colocalisation visible.

1.3.4 Crustacean hyperglycaemic hormone.

The first investigation into the existence of a "diabetogenic" factor in the eyestalk of crustaceans was carried out by Abramowitz *et al* (1944), when it was observed that eyestalk extracts injected into the crab, *Callinectes sapidus*, caused an increase in the blood sugar levels. Experiments showed that 90% of the hyperglycaemic activity present in the eyestalk was found to be associated with the sinus glands. These findings were soon confirmed in other decapod crustaceans (Kleinholz and Little, 1949; Kleinholz *et al*, 1950; Kleinholz *et al*, 1967; Keller, 1969; Kleinholz and Keller, 1973; Leuven *et al*, 1982), in two isopod species (Gersh and Eibish, 1976; Martin *et al*, 1984; Martin *et al*, 1992) and in a stomatopod species (Keller, 1969). From these initial experiments it was determined that CHH had a homeostatic role within the Crustacea and that eyestalk ablation could cause hypoglycaemia. Extirpation of the sinus glands in *Orconectes*

not only causes a decrease of circulating glucose levels, but in addition, abolishes the circadian rhythm of glucose concentration that is normally maximal at night (Hamann, 1974). These experiments demonstrated the role of CHH, not only for homeostatic control of resting glucose levels, but may be required for the elevation of glucose levels at times of physiological need. Clearly, CHH may be instrumental for the production of easily metabolisable energy supplies.

1.3.4.1 Physiological significance and species specificity.

The occurrence of stress hyperglycaemia has been well documented and can be observed in decapods in response to various environmental stimuli including lack of oxygen, temperature elevation and overcrowding. This effect, in the majority of cases, can be abolished by eyestalk ablation (Keller, 1974; Kleinholz and Keller, 1979; Keller and Orth, 1990; Smullen and Bentley, 1992; see also Chapter 5 of this thesis). Dean and Vernberg (1965b) and Salminen and Lindquist (1975) demonstrated that hyperglycaemia and temperature elevation have a positive correlation, although in destalked animals, there is no elevation of haemolymph glucose. Keller and Orth (1990) demonstrated, however, that destalked *Orconectes* are still capable of displaying increased levels of circulating CHH in responce to temperature stress (see Chapter 5). In *Orconectes*, hyperglycaemia occurs in response to anoxia only in intact animals (Keller and Orth, 1990). The effects of stress-induced hyperglycaemia have not been investigated in great detail and many questions remain to be answered. For example, the mortality of the destalked animals under anoxic conditions is no higher than that of intact animals, therefore the importance of CHH for physiological adaptation should be questioned. This will be discussed further in Chapter 5 of this thesis.

Telford (1968) demonstrated that haemolymph glucose titres increased shortly before ecdysis in three species of crab. Maximal levels of glucose in the shrimp, *Penaeus vannamei*, during the intermoult, probably resulted in an accumulation of food reserves during this period of active feeding (Chan *et al*, 1988). Likewise, the gradual decline in glucose titres during late proecdysis, corresponded with reduced feeding. Since the glucose levels are at their lowest just before and after ecdysis, it is unlikely that glucose is essential for either chitin synthesis for the new cuticle, or as a source of energy during moulting. Gwinn and Stevenson (1973) have speculated that in *Orconectes limosus*, the major energy source of glucose is chitin, as it is resorbed by the epidermis before moulting, providing sufficient material for both new chitin synthesis and energy for moulting.

Initial studies on the biochemical structure of CHH, adopted classical purification methods of acetone fractionation, ammonium sulphate precipitation, gel filtration, ion exchange chromatography and preparative gel electrophoresis, using whole eyestalks as a starting material (Kleinholz and Keller, 1973; for review see Kleinholz, 1985). A simpler two step purification by the use of gel electrophoresis and filtration and Sephadex G-50, could be used to purity CHH if sinus glands were used as the starting material (Keller and Wunderer, 1978; Keller, 1981). These approaches show comparative results on the structure and cross reactivity of CHH in a number of crustacean species.

Cross-specificity of CHH has been tested within five infra-orders of the decapod crustaceans by measuring the increased concentration of blood glucose after the injection of eyestalk extracts. The blood glucose concentration of *Nephrops norvegicus* (an astacuran) increased after injections from *Astacus leptodactylus*, as well as from *Carcinus* eyestalk extracts, but the latter effected a lower increase of blood sugar, compared to saline injections. Hyperglycaemic

activity has been identified in all but one of the decapods that have been studied to date, that exception being the anomuran, *Munida rugosa*, whose sinus glands extract showed no hyperglycaemic activity in this species, or in other decapods tested. (Leuven *et al*, 1982; Keller *et al*, 1985). Studies on the electrophoretic mobility of CHH of various species, demonstrated remarkable differences, indicating probable structural diversity of the neuropeptides. (Kleinholz and Keller, 1973; Keller, 1981; Keller *et al*, 1985). It has been found, for example, that crude or partially purified preparations from brachyurans, while highly effective in brachyurans, have little or no hyperglycaemic effect in astacurans, or vice versa. (Kleinholz and Keller, 1973; Keller, 1981).

It should be noted however, that of the four HPLC purified isoforms of CHH from *Homarus americanus*, only three are able to evoke a hyperglycaemic response in a heterologous bioassay with the crayfish, *Orconectes limosus* (Tensen, 1991). In addition, only three of the four HPLC purified isoforms of CHH from *Homarus americanus* produced a hyperglycaemic response in *Astacus leptodactylus*, while all four were active in *Homarus* (Soyez, *et al*, 1990). The study by Leuven *et al* (1982) used crude sinus gland extracts for bioassay experiments and deduced bioactivity at the systematic level of infra-order, however, the results described above indicate that some CHH isoforms may be active only at the species level.

1.3.4.2 Structural aspects.

These early purification methods indicated that CHH is an acidic peptide of 6-7kDa (For review see Keller *et al*, 1985; Kleinholz, 1985; Van Herp, 1988), although recent work shows a variation of molecular weight between different species and CHH isomers. This will be discussed later in Chapter 4 of this thesis. CHH activity has also been associated with a protein of 20.5kDa from the

eyestalks of *Crangon crangon* (Skorkowski *et al*, 1977) although this is considered to be the CHH prohomone. The complete CHH preprohormone sequence for *Carcinus maenas* has been deduced by cDNA cloning (Weidemann *et al*, 1989) This will be discussed later in Chapter 6. The use of HPLC on reverse phase (RP-HPLC) supports have enabled the quick and efficient purification and biological characterisation of CHH from sinus glands extracts instead of whole eyestalks. This method of purification has demonstrated that CHH is polymorphic and that hyperglycaemia, in most crustaceans studied, is induced by at least two peptides with similar molecular weights and amino acid compositions. These can only be separated by their hydrophobic and isoelectric charge responses (Newcomb, 1983; Stuenkel, 1983; Keller and Kegel, 1984; Newcomb *et al*, 1985; Huberman and Aguilar, 1986; Stuenkel, 1986; Huberman and Aguilar, 1988a; Huberman and Aguilar, 1988b; Huberman and Aguilar, 1989; Tensen *et al*, 1989; Soyez *et al*, 1990; Kegel *et al*, 1991; Tensen *et al*, 1991; Huberman *et al*, 1992; 1993; Martin *et al*, 1992; for review see Keller, 1992). HPLC was used to obtain the complete amino acid sequence of the most abundant form of CHH from *Carcinus maenas* and from *Orconectes limosus*, (Kegel *et al*, 1991), as were the partial sequences of the four CHH isomers of *Homarus americanus* (Tensen, 1991; Tensen *et al*, 1991c) and two isomers of the crayfish *Procambarus bouvieri* (Huberman *et al*, 1992, 1993). HPLC fractionation of sinus glands from the terrestrial isopod, *Armadillidium vulgare*, have also resulted in an amino acid sequence for a hyperglycaemic peptide which is strikingly similar to the decapod hormones (Martin *et al*, 1992).

The CHH from the shore crab, *Carcinus maenas* is a 72 amino acid acidic peptide which is N-terminally blocked with a pyroGlutamate residue, an amidated C-terminal and 6 cysteine residues that form three intrachain disulphide bridges (Kegel *et al*, 1991; Tensen, 1991). The sequence similarity shows that these hormones constitute an authentic peptide family which is interspecific. Sequence

homology is 81% between *Orconectes limosus* and *Homarus* CHH/MIH, the latter being described as a moult inhibiting peptide with hyperglycaemic activity (Chang *et al,* 1990). This point has been discussed in section 1.3.3 of this thesis entitled "Moult inhibiting hormone". The homology between *Orconectes limosus* and *Carcinus* CHH's is 61% (Keller, 1992). It is interesting that sequence differences confer striking limitations on interspecific hyperglycaemic activity and explains results obtained by (Leuven *et al,* 1982; Keller *et al,* 1985).

It has been suggested that the described heterogeneity of CHH is caused by the presence of oxidised or non-oxidised forms of the same peptides (Kegel *et al,* 1989; Keller *et al,* 1985). However, the differences between the two bioactive forms of CHH in *Procambarus bouvieri* have been located within the first eight amino acids from the N-terminus (Huberman and Aguilar, 1988a; 1988b; Huberman *et al,* 1993) and both isoforms differ in secondary structure as determined by circular dichroism (Huberman *et al,* 1989). These differences were also demonstrated in the N-terminal section of CHH isoforms of *Orconectes* and *Homarus* (Tensen *et al,* 1989; Tensen, 1991; Tensen *et al,* 1991c) probably between residues 2 and 8. One explanation for the occurrence of isomers is a partial post-translational modification of L-amino acid residues into D-amino acid residues, thus changing the secondary structure in the N-terminal region (Tensen, 1991). Polymorphism has been demonstrated in GIH (Soyez *et al,* 1991), although the amino acid sequence is the same for both isoforms. It has been suggested that the positioning of disulphide bridges may be the cause of the polymorphism due to the alteration of the tertiary structure of the molecules. This would explain differences of bioactivity, chromatophorotrophic behaviour and enzyme cleavage between the isoforms (Soyez *et al,* 1991).

The CHH isomers I and II have a similar sequence to that of the MIH/CHH peptide (Chang *et al,* 1990) and the amino acid composition of the MIH fragment is identical with the C-terminal end sequence of the CHH's. This may suggest a

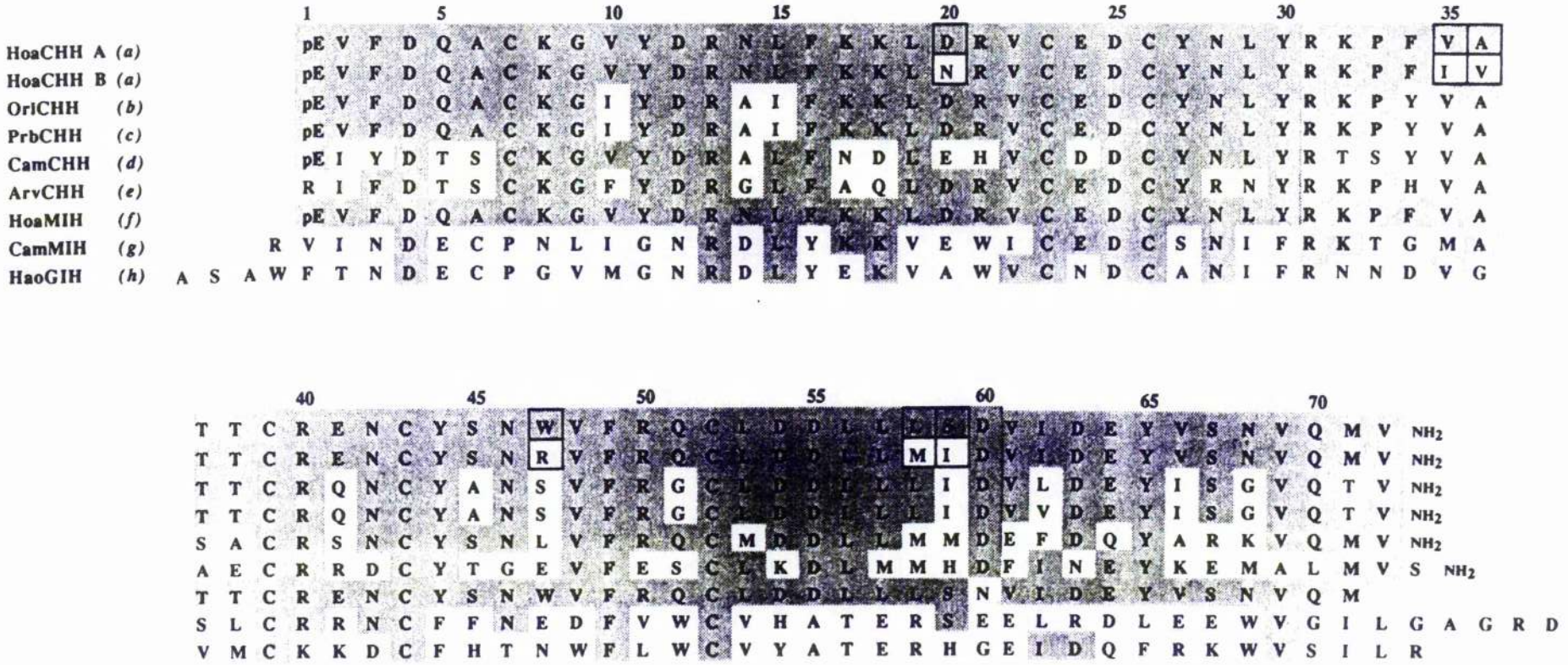
role of CHH in the moult cycle as well as in the control of glycogen metabolism (Tensen, 1991). Receptor binding studies indicate that CHH represses ecdysteroid synthesis which supports this theory (Webster, 1992; 1993). Isomerism of CHH and the comparison of sequences has been review by Keller (1992).

A number of experiments showed the existence of a larger precursor to CHH, notably the purification of a 20.5kDa from the eyestalks of *Crangon crangon* (Skorkowski *et al*, 1977). Pulse chase experiments identified two groups of polypeptides (12-14kDa and 19-21kDa) in the X-organ of the crayfish, *Orconectes virilus*, and the crab, *Callinectes sapidus* and a further peptide (7kDa) in the sinus gland (Andrew and Saleuddin, 1979). It has since been postulated that the 14kDa precursor, pro-CHH, gives rise to one CHH, which is post-translationally modified to yield three different CHH's and one peptide of 4.5kDa, designated peptide H (Stuenkel, 1986). Peptide H has been recognised as CHH precursor related peptide (CPRP) in *Orconectes limosus* and *Homarus americanus* (Tensen, 1991; Tensen *et al*, 1991d). The entire preproCHH has been sequenced by Weidemann *et al* (1989), and Tensen (1991) for review. Recent work suggests that there are two CHH precursors (Tensen, 1991; De Kleijn Van Herp, 1992). It is suggested that the physiological function of the precursors may help to explain the functional and molecular significance of CHH isoforms. The CHH isoforms of *Homarus americanus* had been shown to originate from two different genes (Tensen *et al*, 1991b), as have CHH and MIH from *Carcinus maenas* (Kegel *et al*, 1989; Webster, 1991) and MIH and GIH from *Homarus americanus* (Chang *et al*, 1990; Soyez *et al*, 1991). Table 1.3 shows the comparisons of the primary structures of CHH, GIH and MIH from different crustacean species.

Table 1.3

Primary structure of Crustacean Hyperglycaemic Hormones (CHH), Moult Inhibiting Hormones (MIH) and Gonad Inhibiting Hormone (GIH) from different crustacean species. Hoa: *Homarus americanus*; Orl: *Orconectes limosus*; Prb: *Procambarus bouvieri*; Cam: *Carcinus maenas*: Arv: *Armadillidium vulgare*. *(a)*: Tensen *et al*, 1991a; *(b)*: Kegel *et al*, 1991; *(c)*: Huberman *et al*, 1993; *(d)*: Kegel *et al*, 1989; *(e)*: Martin *et al*, 1993; *(f)*: Chang *et al*, 1990; *(g)*: Webster, 1991; *(h)*: Soyez *et al*, 1991. Conserved residues (related to HoaCHH A) are shaded; residues which differ between HoaCHH A and HoaCHH B are boxed. HoaGIH and CamMIH sequences are shifted in order to optimise the homologies with CHH sequences. (Table 1.3 is a modified version of a table taken from Martin *et al*, 1993).

Hormone (see legend for reference)



1.3.4.3 Immunological studies.

Antisera raised against CHH, to immunospecifically identify the CHH neurosecretory pathway in the eyestalk of the crayfish, *Astacus leptodactylus*, identified approximately 30-35, 50μm perikarya in the MTGXO (Van Herp and Van Buggenum, 1979). The axonal tract to the sinus gland and the axon terminals could be traced in this and other species using the same antisera, although the number of immunopositive cells within the perikarya varied between species (Gorgels-Kallen *et al*, 1982). The use of immunocytochemistry has shown the presence of CHH in the Golgi sacculi and elementary granules in the cell body of *Astacus* and *Carcinus*. This demonstrates that in the sinus gland, the immunoreaction is restricted to one granule type (Jaros and Keller, 1979; Gorgels-Kallen and Van Herp, 1981; Keller *et al*, 1985).

The development of radioimmunoassay (RIA), using antisera raised against *Carcinus*, gave a lower limit of detection of CHH of approximately 10 fmole (Jaros and Keller, 1979; Keller *et al*, 1985). This RIA has been used to assess the cross reactivity of other CHH's to that of *Carcinus*, the distribution of CHH in the CNS and the levels of hormone in the haemolymph under different environmental influences (Martin *et al*, 1984; Keller *et al*, 1985, Keller, 1988; Keller and Sedlmeier, 1988; Keller and Orth, 1990).

The use of enzyme linked immunosorbent assay, (ELISA), has become a standard procedure for the testing of HPLC fractions for immunoactivity (Kallen and Meusy, 1989; Tensen *et al*, 1989; Meusy and Soyez, 1990; Tensen, 1991). The use of a double antibody sandwich enzyme linked immunosorbent assay, (DAS-ELISA), (Voller *et al*, 1979), enabled the measurement levels of circulating haemolymph CHH due to the increased sensitivity of this assay. The circadian rhythmicity of CHH in *Orconectes* was demonstrated using this method (Kallen *et al*, 1990). Hamann (1974) demonstrated a nocturnal peak of glucose, however, the

above method demonstrated that CHH is released at the onset of darkness, two to four hours prior to the maximal peak of glucose. The same process occurs at the beginning of the light period, although the effect is less extreme (Gorgels-Kallen and Voorter, 1985). The circadian rhythmicity of CHH mRNA has been demonstrated in *Orconectes* showing a peak five hours before the onset of darkness while the lowest levels were at the shift from light to dark (Tensen, 1991). It is considered that signals from both the biological clock and the light stimuli act through synaptic connections to CHH synthesising cells, their visualisation by tracing with Lucifer Yellow (Gorgels-Kallen, 1985).

Finally, the localisation of CHH encoding mRNA in the CHH cell somata of eyestalks of *Orconectes* and *Homarus* using a cRNA probe was demonstrated by Tensen *et al*, (1991a).

1.3.4.4 Mode of action and targeting of CHH.

When a sinus gland extract is administered to a test animal, the response follows a characteristic time course. An elevation of blood sugar levels, which are measurable within 10 minutes after injection, a maximum which is reached after about two to three hours, and then, in most cases, a rapid decline of the blood sugar level. (Keller, 1985). The time course for *Nephrops norvegicus* , however, does not appear to follow the same pattern (Smullen and Bentley, 1993; see also Chapter 3). This suggests that hyperglycaemia is caused by a rapid mobilisation of glucose from reserve carbohydrates, rather than more indirectly, by changes of metabolite fluxes through metabolic pathways (Keller and Andrew, 1973; Keller, 1981; Soyez *et al*, 1990). The time course also indicates that CHH activity is not long acting as it may be rapidly inactivated, possibly by internalisation.

Both the hepatopancreas and the muscles contain large reservoirs of glycogen that could be degraded by CHH activity. Parvathy, (1972), showed an increase of glycogen in the hepatopancreas following eyestalk ablation. When hepatopancreas were incubated *in vitro* in CHH, there was a maximal increase of glucose after two hours, a similar time course for the previously observed hyperglycaemic response. Additionally, there was a decrease in the glycogen content of the hepatopancreas. Recently published work by Kummer and Keller (1993) demonstrates the high affinity binding of CHH to isolated hepatopancreatic plasma membranes of both *Carcinus* and *Orconectes*. Other possible target tissues for CHH were investigated by Sedlmeier (1985). It was found that abdominal muscle, hepatopancreas, the heart, integumental tissue and adrenal gland in *Orconectes limosus* , both *in vivo* and *in vitro* all responded with elevated levels of cyclic nucleotides, (cAMP and cGMP), after injection of CHH. However, higher cAMP concentrations were recorded in other tissues after preincubation which may have been caused by handling. Similar results were obtained on *Carcinus* and *Uca* when the hepatopancreas and the claw muscles were injected

The breakdown of the cyclic nucleotides ATP and GTP (adenosine triphosphate and guanosine triphosphate) to cAMP and cGMP (cyclic adenosine monophosphate and cyclic guanosine monophosphate) is catalysed by the synthesising enzymes, adenylate cyclase, (AC), and guanylate cyclase (GC), respectively. However, this dephosphorylation reaction can be forced backwards by phosphodiesterases, which phosphorylate the cAMP and cGMP back to ATP and GTP. This reversible reaction in its normal state remains at equilibrium. The addition of CHH either stimulates AC and GC, or inhibits the cAMP and cGMP phosphodiesterases. Either way, the net result forces the reaction to the dephosphorylated end product. Sedlmeier, (1985), showed that AC stimulation seems more likely and that any increase in phosphodiesterases is probably due to this increase of cyclic nucleotides.

Cyclic nucleotide dependent protein kinases (CNDPK) represent the central part of the proposed pathway. If an increase of cyclic nucleotides is necessary in the breakdown of glycogen to glucose by CHH action, one should also be able to show the activation of cAMP and cGMP dependent protein kinases; within 15 minutes a significant increase in tissues was observed. (Sedlmeier, 1985). The increased circulating cyclic nucleotides are bound to the CNDPK to produce an activated form of the enzyme. In glycogen metabolism, the most important enzymes phosphorylated by these protein kinases are glycogen synthase, phosphorylase kinase and glycogen phophorylase, via phosphorylase kinase. Glycogen synthase transfers glucose from uridinediphospho-glucose to glycogen and exists in two forms; a phosphorylated inactive form and a dephosphorylated active form.

The hyperglycaemic action is mediated via this cascade causing activated CNDPK to phosphorylate the glycogen synthase. This means that the dephospho- form would be converted to the phospho- form and the enzyme subsequently inactivated, thus creating a net glucose increase. The CHH is acting on the glycogen synthase via cyclic nucleotides as a second messenger. Injection of cyclic nucleotides has produced the same response as the CHH (Sedlmeier, 1985).

It appears therefore, that the CHH acts upon glycogen synthase in an inhibitory manner, mediated by a cyclic nucleotide second messenger system. Ablation experiments showing glycogen increases support these findings (Paruathy, 1972).

1.4 Aims of the thesis.

As discussed previously in this introduction, the knowledge of the biology of this species is limited, and in particular that of its endocrinology. The primary aim, therefore, of this research has been to investigate the endocrinology of this species with particular reference to CHH. The initial aims were to investigate the action of CHH in *Nephrops norvegicus* and to compare that action to that of other Crustacea previously investigated by other researches.

In order to compare and contrast the effects of CHH in *Nephrops norvegicus* to other species, it is necessary to isolate and purify the neuropeptide from the sinus glands. The mode of action of the crude sinus gland extracts when injected into the species will only show similarities or differences to other species. The amino acid sequence for *Nephrops* CHH will enable endocrinologists to compare and contrast CHH molecules previously isolated from other species and relate this to the biology and the phylogeny of *Nephrops*. Will the CHH sequence for *Nephrops* be similar to that of other Astacidea, such as *Homarus* and *Orconectes*, or will differences in the mode of action of the peptide be translated in the sequence of the peptide? Antibodies specific to CHH in *Orconectes* and *Homarus,* will be used to investigate the immunospecificity of the *Nephrops* CHH to that of the other species. An immunopositive response to antisera specific to CHH raised in other species, will indicate the possibility of a high degree of homology of sequence of the CHH peptides, especially in the region of the epitope.

Oligonucleotide primers, specific to the *Homarus* CHH sequence, will be used to obtain a nucleotide sequence for the *Nephrops norvegicus* CHH. This will confirm a sequence obtained by the use of biochemical techniques, although PCR will demonstrate that the primers specific to regions of the *Homarus* CHH may show reasonable homology to the same regions of the *Nephrops* peptide. Using

these techniques, it is hoped to demonstrate the expression and/or production of CHH in the developing embryo of *Nephrops*. The culture of ova will give an accurate account of the embryonic development of the species.

Finally, it is proposed to use both biochemical techniques to investigate the physiological implications of CHH on this species. How does *Nephrops norvegicus* adapt to physiological stress and how does this species compare to others within the same class, the Astacidea? The burrowing behaviour of *Nephrops* may suggest that the stresses imposed on the animal are quite different to those imposed on a fluvial species. It is proposed to investigate these differences and demonstrate that the of the role of CHH during physiological stress may be highly specific to a particular species due to the very nature of the species habitat.

Chapter 2.

Materials and Methods.

2.1 Introduction.

During the course of this study, a wide range of experimental techniques have been employed. The aim of this chapter is to present the definitive materials and methods which shall be referred to throughout the remainder of the thesis. This prevents the need for an extensive materials and methods section in each chapter. All chemicals are from the Sigma Chemical Company Ltd. unless otherwise stated.

2.2 Collection and maintenance of animals.

Specimens of the Norway lobster, *Nephrops norvegicus*, were trawled by local fishermen from the Forth/Cromarty region of the North Sea. The depth at which animals were trawled has not been taken into account. The animals were maintained in tanks of running sea water (6-12°C) on a 8:16 hour light:dark cycle with a low light intensity (Light phase at 0800hrs). Lengths of plastic drainpipe were provided as artificial burrows in which animals could seek refuge.

Animals were fed weekly on a diet of *Arenicola marina* or small pieces of fish. Prior to experimentation, however, individuals were starved for a period of four to seven days in order to establish a level baseline of circulating glucose. Males and females in the C-stage of their moulting cycle were used for the homologous bioassay, and males of approximately 30–40mm carapace length, 20–30g in weight were generally selected for experimentation, in order to remove variation due to reproductive state or age of individuals.

Specimens of the fresh water crayfish, *Pacifastacus leniusculus*, were maintained in running tap water between 10°C and 16°C on a 8L:16D photoperiodic regime with a low light intensity (Light phase at 0800hrs). No selection of sex or moult staging were under taken when animals were used for heterologous bioassay.

2.3 Sampling and preparation of haemolymph for hyperglycaemic bioassay.

Animals used for bioassay were starved and kept individually for three to four days prior to experimentation. All tests were commenced in the late morning to minimise the effect of diurnal glycaemic activity as shown by Hauman (1974) and Kallen *et al* (1988, 1990), although *Nephrops norvegicus* appears to have no obvious circadian rhythm for hyperglycaemia (see Chapter 5 of this thesis).

Haemolymph samples were taken by means of a 1ml disposable hypodermic syringe and needle (25 gauge) from the ventral uropod sinus; the handling time for each animal was always less than one minute. 50μl haemolymph samples were taken routinely and aspirated into an equal volume of anticoagulant consisting of 0.1M phosphate buffered saline (PBS), 0.01M sodium citrate and 0.01M ethylenediaminetetraacetic acid (EDTA) (Kallen, 1990). The samples were mixed briefly, centrifuged for 10 minutes at 15,600g (IEC Centra-M microcentrifuge) to remove any particulate matter and then either used immediately in the bioassay or frozen at -20°C for storage.

All injection of eyestalk material, HPLC fractions or control samples, were made into the sinus of the cheliped of the donor animal. Heterologous bioassay using *Pacifastacus leniusculus*, was carried out in an identical manner to the *Nephrops norvegicus* bioassay.

2.4 *In vitro* bioassay for the measurement of haemolymph glucose and hence CHH activity.

Haemolymph glucose was determined by the use of a diagnostic kit (Sigma Chemical company Ltd., procedure No. 510, 1992) which was modified to a micro

assay. The Sigma procedure is based upon the following coupled enzymatic reactions:

Glucose + $2H_2O$ + O_2 —(Glucose Oxidase)→ **Gluconic Acid + $2H_2O_2$**

$2H_2O_2$ + o-Dianisidine (colourless) —(Peroxidase)→ **Oxidised o-Dianisidine (Brown)**

The intensity of the brown colour measured at 425-475nm is proportional to the original glucose concentration. Calibration curves were produced for both the standard glucose kit and the micro version in order to ensure that the two methods were comparable. The following protocol was used for the micro assay. Triplicate 20μl haemolymph/anticoagulant samples were aliquoted into a micro plate (Greiner Labortechnik). 180μl of combined enzyme-colour reagent solution (PGO enzyme and o-dianisidine dihydrochloride) were added to each sample and the plate agitated. Each plate was incubated at room temperature for 45 minutes and absorbance was determined at 450nm (Dynatech MR5000 plate reader). A calibration curve was created in each plate from a stock glucose solution in addition to a negative (blank) and positive (sinus gland extract) control. Glycaemia was expressed in mg glucose.$100ml^{-1}$ haemolymph. Statistical analysis was performed using the Student *t*-test from StatWorks (version 1.2), Cricket Software Inc..

2.5 Eyestalk ablation of experimental animals.

In order that the effects of host CHH activity could be negated from a controlled group of animals and to establish that any increase of circulating haemolymph glucose was the result of endogenous CHH and not from any other metabolic pathway, eyestalk ablation was necessary. As eyestalk ablation removes

major neurosecretory tissue from the animal, it is understandably a stressful operation which results in high mortality. *Nephrops norvegicus*, is highly sensitive to invasive surgical techniques and as a result the mortality following eyestalk ablation in this species is considerably higher than in other crustacean species reported.

In an attempt to reduce this post-operative mortality, animals were placed into containers with a small volume of sea water and slowly cooled to a few degrees above freezing. The animals were removed and the rostral region swabbed with methanol. Standard aseptic technique was adopted and the post-operative site was cauterised with a hot wire and finally dusted with a small quantity of antibiotic powder (Streptomycin). The animals were placed back into the cold sea water which was slowly allowed to return to ambient aquarium temperature. Using this technique, post-operative survival increased from 20% to between 50-60%.

2.6 Sinus gland collection from *Nephrops norvegicus*.

Routine eyestalk ablation was performed on *Nephrops norvegicus* obtained from the west and east coasts of Scotland at the Gleneagles Hotel, Perthshire and the Caledonian Hotel, Edinburgh. The eyestalks were collected from live animals of both sexes, making no note of either sexual maturity or stage of moult and were stored temporarily on ice until they were lyophilised (Genevac SF50) and stored below -20°C. Fresh eyestalks were used additionally from animals stored in the aquarium. Prior to dissection, the eyestalks were rehydrated in ice cold saline and the sinus glands dissected in batches of twenty and immediately re-frozen at -20°C for storage.

2.7 Preparation of sinus gland peptides from *Nephrops norvegicus*.

Routinely, 80-130 sinus glands were homogenised ultrasonically at 0°C in 0.1N HCl (10μl/sinus gland) (MSE Soniprep 150 homogeniser). The resulting homogenate was maintained in a water bath at 80°C for 5 minutes and frozen immediately in liquid nitrogen. The preparation was then subsequently thawed and then centrifuged at 15,600g for 20 minutes (IEC Centra-M microcentrifuge) and the supernatant was stored below -20°C until use.

An alternative method was adopted where sinus glands were homogenised in 1ml of 2M acetic acid (MSE Soniprep 150 homogeniser) for five minutes. The resulting homogenate was centrifuged at 15,600g for 20 minutes (IEC Centra-M microcentrifuge). The supernatant was removed and the pellet resuspended in a further 1ml of 2M acetic acid and centrifuged as before. The resulting supernatant was combined with the initial extraction and frozen at -20°C for storage. The two methods of extraction mentioned above were treated identically following the preparation of the supernatants.

2.8 HPLC of sinus gland peptides.

The supernatant was thawed and the volume was reduced to 175μl by centrifugal evaporation (Genevac SF50) before injection into the HPLC system using a Hamilton syringe. This volume was estimated as between 130 and 300 sinus gland equivalents, depending on the number of sinus glands initially dissected. The chromatographic equipment was a Perkin Elmer series 410 LC pump fitted with 175μl Rheodyne 7125 syringe loading sample injector, UV absorbance was monitored at 214nm using a Perkin Elmer LC-90 spectrophotometric detector which was connected to a Perkin Elmer LCI-100 integrator, the mobile phases being degassed by an initial 15 minute flow of helium.

Analytical chromatography was performed on a Capital Nucleosil C-18 column (5μm particle size, 250mm x 46mm internal diameter) with two differing solvent systems. The first system was a discontinuous linear gradient of n-propanol (Aldrich Chemical Company Inc.) containing 0.1% trifluoroacetic acid (TFA, Fisons) against MilliQ™ water containing 0.1% TFA (pH2.2) at a flow rate of 0.75ml/minute. The second system differed only in that n-propanol was replaced with far UV acetonitrile (BDH) and a flow rate of 1ml/minute was used. All solvents were HPLC grade.

Fractions were collected each minute (Pharmacia LKB Frac-100 Fraction Collector) in Eppendorf tubes siliconised with dimethyldichlorosilane solution (BDH). The fractions were then dried under vacuum (Genevac SF50 Centrifugal Concentrator) and were stored under nitrogen at -20°C until they were required.

2.9 Utilisation of antibody specific for CHH.

The use of antibodies specific to a particular hormone allows for the identification of an immunopositive reaction at a very low concentration. It is a more sensitive expedient than bioassay, enabling fractions from the HPLC to be tested for immunopositivity quickly and using a relatively small quantity of the sample. Anti-*Orconectes* CHH antiserum raised in rabbit was a gift from Professor R. Keller in Bonn and anti *Homarus* CHH antisera raised in guinea pig was from Dr. D. Soyez in Paris.

Both polyclonal antisera, as their name suggests were specific to CHH, however, both were raised from different species, *Orconectes limosus* and *Homarus americanus* and in different species, rabbit and guinea pig. There has been shown to be cross reactivity between the two species and *Nephrops norvegicus* (Leuven,

1982) which enables these antisera to be used for immunochemical detection of *Nephrops* CHH material in a direct ELISA (Enzyme Linked Immunosorbent Assay).

Anti-*Homarus* GIH antisera was an additional gift from Dr. D. Soyez in Paris. This was used to identify GIH (GIH, gonad inhibiting hormone) peaks from the HPLC chromatogram by testing the fractions in exactly the same manner as discussed in section 2.11, although the primary antibody is anti-*Homarus* GIH rather than anti-*Orconectes* CHH. In addition, the anti-*Homarus* GIH used for the direct ELISA was tested with two positive controls; sinus glands from both *Nephrops norvegicus* and from *Homarus gammarus*. There appeared to be no difference of binding capability of the GIH antisera to either of the positive controls.

2.10 Purification of anti-*Orconectes* CHH antiserum.

A Protein A Sepharose column was prepared with 100μl Protein A Sepharose in a 1ml disposable syringe barrel plugged with polyester wool which was washed with 10ml of 0.1M phosphate buffered saline (PBS). In order to clean up the anti-*Orconectes* CHH, the antiserum was resuspended in 500μl of PBS, applied to the column and the eluant recycled a number of times. The column was then washed with 5ml of PBS. The column was eluted with 50mM gly HCl, pH 2.8 and 200μl fractions were collected into Eppendorf tubes containing 100μl of TRIS HCl pH 8.0 (0.5M) which were read at 280nm (Cecil CE599 spectrophotometer) for protein absorbance. The anti-*Orconectes* CHH antiserum was eluted in the first 800μl and these fractions were pooled, diluted to a final volume of 10ml and dialysed against PBS for 28hrs at 4°C.

A protein concentration of $0.302 mg.ml^{-1}$ was estimated by spectrophotometric determination at 280nm. 5ml of this primary antiserum was stored frozen at -20°C. The remaining 5ml was diluted to 15ml and divided into 1ml

aliquots each containing 100μg.ml^{-1} of antiserum which was also stored at -20°C. This primary antibody solution was diluted 1:10 and then used for ELISA. The anti-*Homarus* GIH antisera that was donated by Dr. Soyez did not require purification by the use of Protein A Sepharose.

2.11 Calibration of the ELISA.

10 sinus glands were dissected from freshly sacrificed *N. norvegicus* and homogenised ultrasonically in 2ml of ice cold PBS (MSE Soniprep 150). 200μl of the resulting homogenate, approximately equivalent to 1 sinus gland was diluted in 200ml of PBS. The assay was prepared in two 96 well microtitre plates (Greiner Labortechnik, ELISA).

100μl of PBS was pipetted into each well and then 200μl of the antigen was added to the second column leaving the first as a blank. 100μl was then removed and pipetted into the next column to give a 50% dilution. A standard curve was therefore prepared for each plate. The antigen was left for 28hrs at 4°C and was then washed three times by repeated addition of 300μl of 0.1M PBS containing 0.1% polyoxyethylene sorbitan monolaurate buffer (PBS-Tween). When an ELISA was used to test fractions for GIH activity, at this stage in the procedure a 3% powdered milk solution was added to each well in order to prevent non-specific binding of the GIH antisera.

100μl of PBS-Tween was put in each well and 200μl of the primary antibody was added to the first row in a 1:2.5 dilution antibody to PBS-Tween, again leaving the first row as a blank. 100μl was then removed and pipetted into the next row, which was mixed and 100μl again removed and added to the next row thus creating an antibody standard curve. This was left at 20°C for 2 hours. Each well was then washed three times with 300μl of PBS-Tween and incubated for 1

hour at 20°C with 200μl of anti-rabbit biotinylated secondary antibody (Amersham) diluted 1:1000 with PBS-Tween.

After three additional washes with 300μl of PBS-Tween, 200μl of biotinylated Streptavidin peroxidase (Amersham) diluted 1:1000 with PBS-Tween was pipetted into each well and left to incubate for 1 hour at 20°C. The wells were then washed three times with 300μl PBS-Tween. Finally the enzymatic activity was assessed by the addition of 200μl of staining reagent (5.14ml of 0.2M Na_2HPO_4 and 4.86ml of 0.1M citric acid in 20ml containing 8mg of o-phenylenediamine and 10μl of H_2O_2) into each well. The plate was read every 5 minutes at 450nm (Dynatech MR5000 plate reader) until an O.D. of approximately 0.5 was obtained at which point the reaction was stopped by adding 100μl of 3M H_2SO_4. The plate was read again at 490nm.

2.12 Use of ELISA on HPLC fractions.

From the chequer board design described above, it was possible to use as little as 0.5 sinus gland equivalents for a direct ELISA, and a dilution of primary antibody of 1:1000 gave an optimum reaction. The lyophilised HPLC fractions were reconstituted in a relevant volume of PBS and 0.5 sinus gland equivalents were pipetted in triplicate into the micro plates. The procedure discussed in section 2.11 was adopted, using 200μl of primary antibody. However, it was observed during subsequent experimentation that it was the antigen that was limiting to the reaction and therefore it was possible to dilute the anti-*Orconectes* CHH antisera considerably more than is described here.

Occasionally, the ELISA were carried out at 37°C. In so doing the timing for each step could be considerably reduced. Antigen binding could be obtained in

two hours, while each resulting step could be carried out with an incubation of 30 minutes instead of the usual 1-2 hours.

2.13 Use of ELISA on various stages of egg development.

Groups of 20 ova were collected at a number of different developmental stages and were homogenised ultrasonically on ice (MSE Soniprep 150 homogeniser). Ova collected were estimated to be at 5%, 24%, 50% and 90% development (Helluy and Beltz, 1991). The resulting homogenate was centrifuged at 15,600g for 20 minutes (IEC Centra-M microcentrifuge) and the supernatant was collected and frozen at -20°C for storage. The thawed supernatant was then applied to a 96 well microtitre plates (Greiner Labortechnik, ELISA) and a direct ELISA was carried out as described above in section 2.11.

Anti-*Orconectes* CHH and anti-*Homarus* GIH antisera were used to assess at which stage of development a peptide was present that would bind specifically to a particular antisera. Following the binding of antigen, a 3% powdered milk solution was added to each well in order to prevent non-specific binding of the antisera. It was observed in previous experiments that the colour reaction that occurs during the ELISA takes place at a considerably slower rate for the ova than for the HPLC fractions. One sinus gland equivalent from an immunopositive HPLC fraction was used as a positive control and was serially diluted in order that an estimation of peptide concentration could be calculated.

2.14 Protein determination.

Peptide content of sinus glands, selected fractions and pre-HPLC supernatant, was quantified by the bicinchoninic acid method and related to peak area on the chromatogram. The measurement of protein using bicinchoninic acid

was a micro modification from Smith *et al* (1985). Reagent A is essentially as described by Smith *et al* (1985) except that potassium sodium tartrate was used instead of disodium tartrate.

Reagent A:

1.7% sodium carbonate

0.16% potassium sodium tartrate

0.4% sodium hydroxide

0.95% sodium hydrogen carbonate

1.0% bicinchoninic acid

All dissolved in distilled water to give the final concentrations above and the pH adjusted with sodium hydroxide to 11.25. The reagent is stable indefinitely at room temperature.

Reagent B:

4% copper sulphate pentahydrate in water

Standard Working Reagent (SW-R): 100 volumes of reagent A + 2 volumes reagent B (to be prepared as required - although the original paper suggests that the working reagent is stable for one week).

A bovine serum albumen (BSA) standard curve was created by double diluting $1mg.ml^{-1}$ solution across a micro plate (Greiner Labortechnik). Standards, samples and blanks are made to 12.5μl with distilled water in a micro plate (Greiner Labortechnik). 20 volumes (i.e. 250μl) of SW-R are added to each well and the plate sealed with Nescofilm (BDH) and incubated at 60°C for 60 minutes. Finally, optical densities are measured using a Dynatech MR5000 plate reader using a filter with a wavelength of 550nm

2.15 The use of polyacrylamide gels in the presence of sodium dodecyl sulphate (SDS-PAGE) for molecular weight determination.

A modification of the discontinuous procedure used by Schägger and von Jagow (1987) was adopted to estimate the molecular weight of peptides separated using the HPLC system described in section 2.8. For the visualisation by either Coomassie Brilliant Blue R (Neuhoff *et al*, 1988) or by the silver staining method of Bloom *et al* (1987) it was determined that between 4-8 sinus gland equivalents from the HPLC fractions gave the most suitable concentration.

Aliquots of the fractions which were immunopositive were dried down (Genevac SF50) and resuspended in the SDS sample buffer. Single sinus glands were used as an additional marker, although their relatively high protein content tended to overload the gel. The buffer varied from that of Schägger and von Jagow (1987) in that 2-Mercaptoethanol was replaced with 10mM DTT (Dithiothreitol - Bio-Rad). The samples were warmed to 60°C for 30 minutes and mixed before loading onto the gel. The gel differed from the literature in that the percentage of cross linker C (N, N'-Methylene-bis-Acrylamide) was 2.6%, a ratio of 36.5:1 acrylamide to N, N'-Methylene-bis-Acrylamide. The gel was poured in three separate parts and layered with butan-2-ol between each part with a final thickness of 0.75mm. The Bio-Rad Mini Protean II System was used to run the gel which was finally fixed in a solution containing 12.5% TCA (Trichloroacetic acid), 3% sulphosalicylic acid and $50\mu l \cdot 100ml^{-1}$ of a 30% formaldehyde solution. Molecular weight markers (5-10μg) were run beside the samples in order to estimate the molecular weight of the proteins therein. The weights of the markers ranged from 2,500-17,000 and their visualisation was by the silver staining method of Bloom *et al* (1987) and the resulting gels were photographed and developed on a high contrast paper.

2.16 Capillary electrophoresis of purified CHH immunoactive fraction.

Fifteen sinus gland equivalents of the purified CHH immunoactive HPLC fraction was lyophilised and reconstituted with 20μl of a 10mM phosphate running buffer, pH2.5 and applied to the Bio–Rad, BioFocus 3000 capillary electrophoresis system. A running buffer consisting of a 50mM phosphate buffer, pH2.5, was used and the electrophoretic conditions were set at a 15kV constant voltage with the polarity running from positive to negative. The sample was pressure injected for 4 seconds at 5 p.s.i. at 20°C onto a 24cm x 25μm coated capillary (Bio–Rad). Wavelengths between 200nm and 300nm were continuously monitored using the multi wavelength facility incorporated within the BioFocus 3000.

2.17 Slide and whole mount staining with PAF (paraldehyde fuchsin) solutions.

The following staining technique was used for the staining of neurosecretory material in both whole mount dissection of eyestalks and also for wax sections. PAF stains neurosecretory tissue deep purple. The following protocol was adopted.

Preparation of paraldehyde fuchsin stain is shown below.

60% alcohol	100 Ml
Basic fuchsin	0.5 Ml
Paraldehyde	1 Ml
Concentrated HCl	1.5 Ml

(a) Oxidising solution of performic acid

98% formic acid - 10 parts

30% H_2O_2 - 1 part;

Mix, cover, leave for 2 hours.

(b) 25% PAF in 70% alcohol, plus 1% glacial acetic acid.

(c) Acid alcohol - 1% HCl in 95% alcohol.

Procedure for PAF staining for whole mount.

(1) Fix eyestalk in sea water Bouin's fixative fluid overnight.

(2) Wash well in 70% alcohol (several changes; at least 24 hrs).

(3) Oxidise in performic acid - 3-6 Hrs.

(4) Wash well in distilled water (several changes; at least 24 hrs).

(5) 70% alcohol.

(6) Stain in PAF - 1.5-2 hrs.

(7) Wash well in 70% alcohol - 15 minutes.

(8) Differentiate (decolourise background tissue) in acid alcohol.

(9) Dehydrate in absolute alcohol - 2 changes, five minutes each.

(10) Xylene - 5 minutes.

(11) Clear in methyl benzoate/methyl salicylate (50:50 mixture).

(12) Permanent preparations by returning to xylene and mounting in Depex.

For sectioning of material, the tissue was dehydrated through an alcohol gradient with several changes of each. The tissue was perfused and embedded in paraffin wax with a melting point of 56°C. Sections were made between 5μm and 8μm thick and mounted on grease free slides and stained with PAF as described above.

2.18 Dissection and staining using Lucifer Yellow.

An eyestalk dissection of the sinus gland and associated axon tract was made under saline, taking care not to damage the tissue surrounding the XOSG complex. Once dissected, a 45° cut was made 3/4 of the way across the axon and a small drop of distilled water was introduced to swell the cut opening. The whole dissection was then suspended in Lucifer yellow for 24 hours at 4°C. The preparation was then washed several times in saline to remove any excess Lucifer yellow, and then cleared in methyl salicylate for at least 24 hours.. The stained nerves were then viewed under UV light and then was mounted and viewed under a confocal laser scanning microscope.

2.19 Rearing and development of eggs from *Nephrops norvegicus*.

Female berried ***Nephrops norvegicus*** were obtained from mid September/October until March and were maintained as discussed in section 2.2. The eggs are attached to the ventral pleopods of the female animal, who circulates water over the eggs by the gentle movement of the limbs. The animals were maintained in the aquarium, however, if the female appeared unhealthy or died the eggs were carefully stripped from the pleopods using fine forceps and transferred into a inverted plastic bottle containing TFSW. An air stone was inserted through the base of the bottle so that the air gently circulates the eggs, simulating the movement of the pleopods. The combination of the central air flow and the angled sides of the bottle prevented the eggs them from resting on the bottom and therefore reduces the risk of bacterial infection brought about by stagnation.

The egg bearing females with the youngest eggs were selected, the earliest developmental stage observed in the eggs was a cleavage stage. The approximate date of extrusion was estimated by from the appearance of the first eye pigmentation.

Papers by Herrick (1895), Templeman (1940) and more recently Helluy and Beltz (1991) estimate that for the lobster, *Homarus americanus*, the period from extrusion to the first appearance of eye pigment was predicted to be 21 days. Assuming that *N. norvegicus* follows a similar time course, then extrusion would be 21 days prior to the appearance of eye pigmentation at 12-13°C. Ten live eggs were examined every two to three days until after a month when the eggs were assessed weekly. Once the heart was visible, its beat was confirmed for each embryo before measurements were made Three measurements were made using an eye piece graticule across the diameter of the egg in order to obtain an accurate average value and in addition to this, measurements of the length and width of the developing eye was made as this can be used as a assessment of embryo development (Perkins, 1972). A description of the eggs was also made as visual and behavioural changes are an important indication of development.

2.20 Preparation of total RNA from eyestalks of *Nephrops norvegicus*.

Throughout the preparation of RNA, disposable gloves were always worn in order to prevent the transfer of RNases from the hands to the preparation and all baked glassware and plasticware was autoclaved and siliconised with dimethyldichlorosilane solution (BDH).

A total of 40 optic ganglia (approximately 1g) were dissected from the eyestalks of freshly sacrificed animals and placed into a pre-chilled 50ml disposable centrifuge tube containing guanidinium thiocyanate/b-mercaptoethanol (15.3ml guanidinium buffer + 1.7ml b-mercaptoethanol). This was homogenised immediately on ice using the Polytron homogeniser with a large probe for 20 seconds. Before use, the probe of the homogeniser was cleaned with 0.2% SDS and then rinsed with DEP treated (diethyl pyrocarbonate) water and pre-chilled in ice

cold DEP water. To each 17ml of homogenate 23ml of 6M lithium chloride (LiCl) was added and mixed using a 10ml syringe and blunt 18G needle in order to shear the high molecular weight DNA. This was transferred to a 50ml poly-propylene centrifuge tube and allowed to precipitate overnight at 4°C.

The following day the precipitate was spun down at 10,000g (JA 13.1) for 90 minutes in 15ml Corex tubes at 4°C and the supernatant discarded. The insides of the tube, wiped carefully to remove any remaining supernatant and then the pellet was raised in 10ml of 3M LiCl as a wash using a 21 gauge needle and then transferred to clean Corex tubes. Another 5 ml of 3M LiCl was added and mixed, giving a total of 15 ml of 3M LiCl. This was centrifuged again at 10,000g for 60 minutes at 4°C. The supernatant was discarded, the tubes wiped and the pellet then raised in 4 ml of TNESDS (0.1% SDS, 10mM Tris. HCl pH8.4, 1mM EDTA, 150mMNaCl) at room temperature. Proteinase K was then added to give a final concentration of 200-400$\mu g \cdot ml^{-1}$ and the tubes incubated at 37°C for 30 minutes. The resulting digest was then extracted twice with an equal volumes of phenol:chloroform:isoamyl alcohol (1:1:24) and finally vortexed and centrifuged at 9,000rpm 20°C in order to separate the aqueous and organic phases. The two aqueous phases were combined and extracted once with chloroform to remove residual phenol from the aqueous phase, mixed and centrifuged for 5 minutes at 9,000rpm (JA 13.1) at 20°C. The RNA was precipitated in Corex tubes by the addition of 0.1volumes of 3M sodium acetate and 2.5 volumes of absolute ethanol at -20°C overnight. The RNA was pelleted by centrifugation at 10,000g (JA 13.1) for 20 minutes, the precipitate drained and the resultant pellet washed with 3ml of ice cold 70% ethanol. Centrifugation at 10,000g (JA 13.1) for 15 minutes resulted in a pellet which was vacuum dried at 20°C. This was raised in 1 ml DEP water and heated at 65°C, in order to aid dissolution and denature and then cooled quickly on ice. In order to quantify and check the purity of the RNA, the absorbance at 260 and 280nm was measured on a U.V. spectrophotometer, the ratio of absorbancies

should give a value of approximately 2 for pure RNA. ($40\mu g.ml^{-1}$, RNA = 1 OD_{260} 1cm,ml).

2.21 Preparation of total RNA from eggs of *Nephrops norvegicus*.

Approximately 1g of *Nephrops* eggs were washed with 0.5% Triton X-100 and placed into a pre-chilled 50ml disposable centrifuge tube containing 10 volumes of buffer. This was homogenised immediately on ice using the Polytron homogeniser for 20 seconds. The probe was chilled and cleaned prior to use as described above in section 2.20.

The homogenisation buffer consisted of:

50mM NaCl

50mM Tris.Cl (pH 7.5)

5mM EDTA (pH 8.0)

0.5% SDS

$200\mu g.ml^{-1}$ proteinase K

The homogenate was occasionally mixed during a 1 hour incubation at 37°C. The resulting homogenate was then extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol and finally vortexed and centrifuged at 5000g (JA 13.1) at 20°C in order to separate the aqueous and solvent phases. The two aqueous phases were then combined in 15ml Corex tubes and the RNA and DNA were precipitated by the addition of 0.1 volumes of 3M sodium acetate (pH 5.2) and 2.5 volumes of ethanol for 2 hours on ice. The precipitate was pelleted by centrifugation at 5000g (JA 13.1) for 20 minutes, vacuum dried at room temperature and then raised in 1ml of DEP water. This was mixed with an equal volume of 8M LiCl which selectively precipitates RNA and stored at -20°C overnight.

The RNA was recovered by centrifugation at 10,000g for 30 minutes at 4°C and the supernatant carefully discarded. The pellet was washed in ice cold 70% ethanol, recentrifuged for 10 minutes, the supernatant discarded and the pellet vacuum dried at room temperature. The pellet was resuspended in 1ml of DEP water, reprecipitated with 3 volumes of ice cold absolute ethanol and stored at -20°C. The RNA yield was quantified at 260:280nm as described above.

At this point, the pellet was a deep purple colour and was considered to be contaminated with a natural dye. Although this appeared not to effect the RNA quantification, it was thought that it may effect cDNA synthesis and therefore attempts to remove the colour were made. This method is described in chapter 7.

2.22 cDNA synthesis of both eyestalk and egg total RNA.

The synthesis of oligonucleotide primers was carried out at the Catholic University, Nijmegen, by Tensen *et al* (1991) (A gift from F. Van Herp, Nijmegen) and were based on the partial amino acid sequences which are identical between all four CHH's purified from *Homarus americanus*. The amino acid and oligonucleotide sequences of the three fragments used for cDNA synthesis can be found in Tensen (1991) and Tensen *et al* (1991b). These primers are homologous to nucleotides 1-23, 64-86 and 202-218 (P1, P2 & P3) for the *Homarus* CHH and were used in the Polymerase Chain Reactions (PCR) detailed below.

In order that the primers used in the PCR were at equal concentrations, the concentration of each of the oligonucleotide primers was necessary. This was confirmed by determining their molar coefficients (Appligene catalogue, 1992-93). This was calculated by the following formula:

$$\text{em oligo} = 2(\text{em dAT} + \text{em dTG} + \text{em dGC}) - \text{em dT} - \text{em dG} = 3.9 \times 10^4$$

i.e. where em = the molar extinction coefficient. 1 mole of the ATGC sequence will give an OD_{260} of $3.9x10^4$ when solubilised in 1ml.

The molar extinction coefficient values of single deoxynucleotides has been calculated as:

dA 15.4×10^3

dC 7.4×10^3

dG 11.5×10^3

dT 8.7×10^3

The molar extinction coefficient values for each deoxydinucleotides (x 10^3) are:

First deoxynucleotide	Second deoxynucleotide			
	dA	dC	dG	dT
dA	13.7	10.6	12.5	11.4
dC	10.6	7.3	9.0	7.6
dG	12.6	8.8	10.8	10.0
dT	11.7	8.1	9.5	8.4

2.23 First strand synthesis.

6μl of DEP water, 1μl of 0.5$\mu g \cdot \mu l^{-1}$ oligo $dT_{(15)}$ primer and 4μl of RNA were added to a 1.5ml Eppendorf tube and heated to 70°C in order to denature the RNA for priming by the oligo $dT_{(15)}$. The reaction mixture was cooled on ice in order to anneal the oligo dT and the following was added:

5 x first strand buffer	4μl
0.1M DTT	2μl
10mM dNTP's	1μl
Superscript reverse transcriptase (BRL)	2μl
(P^{32}) α dCTP	2μCi
Final volume	10μl

The reaction mixture was incubated at 37°C for 1 hour in a dry block. In order to assess the yield of mRNA first strand synthesis, the extent of P^{32} incorporation into the first strand cDNA was determined by TCA precipitation. Duplicate 0.2μl aliquots of the first strand reaction mixture were spotted onto two glass fibre filters. One filter was dried under an infra-red lamp and the other was washed three times with 10% TCA, 0.2% pyrophosphate. The latter was dehydrated with 3 washes of absolute ethanol, dried and each piece was placed into a scintillation tube and the counts per minute (cpm) measured.

Once the successful synthesis and yield of first strand cDNA had been assessed the remainder of the reaction mixture was made up to 100μl with DEP water and extracted with an equal volume of phenol. The first strand (contained within the aqueous layer) was then co-precipitated with 2μg of yeast tRNA (1$\mu g \cdot \mu l^{-1}$), to act as a carrier for the cDNA, and 0.5 volumes of ammonium acetate (7.5M) and 4 volumes of -20°C absolute alcohol was added and precipitate stored for 1 hour at -20°C. The precipitate was then pelleted by centrifugation for 15 minutes at 13,000g (JA 18.1) and raised in 20μl of TE (10mM Tris.HCl pH 8.4, 1mM EDTA).

2.24 Amplification of cDNA by PCR.

The oligonucleotide primers used for the PCR had their individual Tm calculated as 64.0°C for the 5's P1, 66.6°C for the 3'a P2 and 52.1°C for the 3'a P3 (Gene Jockey Sequence Processor, Biosoft). The Tm is the temperature at which 50% of double stranded molecules become single stranded. The Tm for P3 is lower due to its smaller size compared to the other primers. According to Tensen, (1991) and Tensen *et al* (1991b), a reaction between P1 and P2 results in a 100bp product, while that between P1 and P3 produced a 230bp product.

The reaction conditions for the PCR are shown below:

DEP water	34.8μl
10 x Buffer	5μl (1.5mM $MgSO_4$)
cDNA	5μl
100pM P1	1.2μl
100pM P2	3μl
10mM dNTP's	1μl
Total volume	50μl

(1 x PCR buffer containing 2.5 units Taq polymerase)

The reaction mixture, excluding the Taq polymerase, was layered with 50μl of mineral oil and placed into a Techne PH3 Thermal Cycler. In order to improve the specificity of the reaction, a hot start was utilised. The reaction was heated to 92°C for 2 minutes in order to fully denature the cDNA and then cooled to 85°C. Under these conditions the primer will anneal with increased specificity to the cDNA and will thus reduce the priming of any non-specific product during the first round of amplification.

Following this hot start, 5μl of 1 x PCR buffer containing 2.5 units of Taq polymerase (Amplitaq, Perkin Elmer/Cetus) was added below the mineral oil. The reaction was enhanced by cooling to the calculated Tm for the primers used i.e. the lowest Tm between the two primers. For P1 and P2 denaturation occurred at 94°C for 1 minute, annealing at 60°C for 2 minutes and extension at 72°C for 2 minutes for 35cycles. The final extension was at 72°C for 10 minutes in order to add an A base onto the 3' end for cloning. For P1 and P3 the annealing temperature was carried out at 45°C, 47°C or 52°C for 45 cycles in order to optimise the reaction (see chapters 6 and 7).

25μl of the PCR product was added to 2.5μl of 10 x loading buffer containing 5% bromophenol blue and 2.5μl of 10 x electrophoresis buffer. Resolution was by electrophoresis on a 3.5% tris-borate-EDTA buffered (Nusieve) GTG agarose gel stained with 10μl·10mg·ml^{-1} ethidium bromide. The product size was compared with known standards (Ø x 174/ Hae III) and photographed on a U.V. transluminator.

The DNA concentration from the gel was estimated and removed from the agarose using a Spinex column. The following ligation reaction was carried out:

PCR product	(10-50ng)
TA cloning vector pCR cloning vector	100ng
10X ligation buffer	2μl
T4 DNA ligase	4—8 Weise units
DEP water to make total volume to 20μl.	

The ligation reaction was incubated for 16 hours at 12°C and then the T4 DNA ligase was inactivated at 65°C for ten minutes. The cell were transformed with 100ng of ligated vector following the Invitrogen TA cloning kit protocol.

Chapter 3.

The Sinus Gland of *Nephrops norvegicus* : CHH induced hyperglycaemia.

3.1 Introduction.

In the Crustacea, the occurrence of rapid hyperglycaemia following the injection of eyestalk extracts into a host animal has been well documented. Abramowitz *et al* (1944) first demonstrated this phenomenon in the crab, *Callinectes sapidus*, and identified the sinus gland as the major source of this hyperglycaemic activity. The bioactive product of the sinus glands was first shown to be of a proteinaceous nature due to the action of proteolytic enzymes (Kleinholz *et al*, 1967) and was identified further as having a molecular weight of approximately 7kDa (Keller, 1973). The determination of the amino acid composition of CHH has shown that the peptide displays structural differences between species, this species specificity has been demonstrated by investigating interspecific hyperglycaemic activity (Kleinholz and Keller, 1973; Leuven *et al*, 1982; Keller *et al*, 1985); see Chapter 1 for further details. Recent elucidation of CHH amino acid and nucleotide sequences from various species has further clarified the nature of the interspecificity of the peptide (Kegel *et al*, 1989; Chang *et al*, 1990; Kegel *et al*, 1991; Tensen *et al*, 1991; Huberman *et al*, 1992; Martin *et al*, 1992).

The interspecific nature of CHH was utilised for heterologous bioassay development during this present study. Due to the high inter-animal variation when glucose bioassays were carried out on *Nephrops norvegicus*, initial bioassays were adopted first on the crayfish, *Pacifastacus leniusculus*. The between animal variation in this astacidean was negligible and therefore proved to be an effective animal for initial bioassay of sinus gland extracts and HPLC fractions. As *N. norvegicus* is apparently susceptible to stress hyperglycaemia, experiments which involved repeated sampling and handling were designed in order that any increase of glycaemia could be attributed only to CHH and not to stress.

Before the purification of CHH from *Nephrops* could be initiated, the sinus glands of the species had to be identified and differentiated from other eyestalk

structures. Initial dissections of the eyestalk of *Nephrops* indicated that the collection of sinus glands from this species could prove to be difficult. Unlike the sinus glands of other species such as *Homarus*, *Carcinus*, *Pacifastacus* and *Galathea*, those of *Nephrops* were not discrete blue/white structures, but were pale in colour and had what appeared to be a highly digitate dispersed structure. In addition, pigmentary material was situated in close proximity to the sinus glands thus preventing their ready identification during dissection.

The procedure adopted for the purification of CHH in this study necessitated a reliable bioassay for the measurement of haemolymph glucose. In addition, the time course measurement of CHH within *Nephrops norvegicus* was essential as maximal glycaemia should be measured in order to negate resting or stress related glycaemia. It was necessary to determine the time duration for the maximal activity of the CHH in order that any haemolymph sampling for bioassay could be made at the maximum effect of the CHH on the animal. The most effective dose of sinus gland equivalents from HPLC fractions should be administered to the test animal to enable an accurate identification of active fractions, therefore, a dose response effect of sinus gland injection has been described. Finally, development of the bioassay was made to provide a quick and efficient measurement of haemolymph glucose.

3.2 Materials and Methods.

3.2.1 Dissection of eyestalks of *Nephrops norvegicus* and the comparison of sinus glands from this and other species.

Animals were maintained as described in Chapter 2. Sinus glands were obtained from both fresh and stored eyestalks. The stored eyestalks were removed from fresh animals and stored as described in Chapter 2. There was considerable difference in the nature of the eyestalk tissue between stored and fresh material and

this often created problems when the sinus glands were collected for peptide extraction. Fresh eyestalks were dissected sagittally along the mid dorsal line and the exoskeleton of the eyestalk was then separated. The sinus gland is a blue/white iridescent structure on the dorsal lateral portion of the optic ganglia, at the level of the medulla interna and the medulla externa. The gland lies superficially immediately under a layer of connective tissue and muscle which is associated with eye movement (Laverack pers. comm.). In fresh material, it was occasionally possible to observe the axon tract extending from the sinus gland through the neuropil. Figure 3.1 illustrates the difficulty of sinus gland collection from *Nephrops norvegicus*, especially when compared to the *Homarus gammarus* sinus gland (figure 3.2).

The dissection procedure was different in stored (thawed or rehydrated) eyestalks. Due to the freezing, thawing and rehydration of the optic ganglia, the tissue was easily dissociated and fragmented. In addition, the consolidated structure of the pigmented ommatidia, which retains its' structural integrity in fresh material, breaks up, covering the dissection with dark and light granular pigmentary material. In either case, the location of the sinus gland becomes extremely difficult and laborious. Consequently, a different method was developed for the dissection of stored eyestalks. The rehydrated optic ganglia was grasped with a pair of fine forceps through the cut ends of the eyestalk and gently pulled out of the exoskeleton through the same cut end. Much of the connective tissue between the optic ganglia, the exoskeleton and the ommatidia had been destroyed during the freezing action and therefore, the ganglia could easily be removed from the eyestalk, while the pigmented tissue remained inside the eyestalk. Although this dissection was quicker than the previous method, the visualisation of the sinus gland through the connective tissue was complicated by the loss of colour and distinct structure of the gland, thus making them less conspicuous.

3.2.2 Slide staining of neurosecretory material with PAF (paraldehyde fuchsin) solutions.

Due to the difficulty of identification of the sinus glands of *Nephrops* during dissection, their position in the eyestalk was originally determined by the longitudinal sectioning and staining of the eyestalk with PAF (see Chapter 2), which displayed the neurosecretory tissue therein, *i.e.* the MTGXO-SG system (see figures 3.3 to 3.7). This method was used to visualise not only the sinus glands, but in addition, the axon tract and it's origin, the perikarya that constitute the X-organ. Attempts were also made to stain the XOSG complex and identify any nerve innervation by micro-injection with Lucifer Yellow, a similar method used by Gorgels-Kallen (1985). As the X-organ could not be visualised by dissection, initial attempts to stain the XOSG complex were made by the use of micro-electrode injection through the axon tract, rather than into the CHH perikarya as described by Gorgels-Kallen (1985). Further attempts were made using the method described in Chapter 2.

3.2.3 Determination of haemolymph glucose and hence CHH activity.

Haemolymph glucose was determined using a diagnostic kit (Sigma Chemical company Ltd., procedure No. 510, 1992), which was modified to a micro assay. Since the normal Sigma procedure was slow and laborious and where many haemolymph samples were to be measured routinely, a quicker, more accurate and cheaper method was necessary. The following protocol was used for the micro assay. Triplicate samples (20μl) of haemolymph/anticoagulant were aliquoted into a micro plate (Greiner Labortechnik). 180μl of combined enzyme-colour reagent solution (PGO enzyme and o-dianisidine dihydrochloride) was added to each sample

and the plate agitated. Each plate was incubated at room temperature for 45 minutes and absorbance was determined at 450nm (Dynatech MR5000 plate reader). A calibration curve was created for each plate from a stock glucose solution in addition to a negative (blank) control. This method allowed greater accuracy since many replicate means could be performed and the absorbance determined at identical times. This eliminates any errors from the continuation of the enzyme reaction which could create intra-sample variation.

A series of calibration curves were produced in order that both the normal and micro bioassay could be demonstrated as resulting in comparable estimates of glucose concentration, both with known glucose standards and haemolymph glucose of individual animals. The haemolymph glucose of individual animals was determined both by the normal bioassay and by the plate incubated bioassay, the methodology for which is described in Chapter 2 and above.

3.2.4 Bioassay development.

Animals that were used for bioassay were stored and treated as described in Chapter 2. For each experiment controls were used consisting of twice filtered sterile sea water as a negative control and crude sinus gland extracts as a positive control. All bioassays were carried out on both *Nephrops norvegicus* and *Pacifastacus leniusculus*, however, only data for *N. norvegicus* are shown. As described previously, this was necessary as *N. norvegicus* displayed a large inter-individual variation and the animals' frailty often resulted in high mortality during bioassay experiments. It should be noted that the results of any bioassay have been indicated graphically as "Change in Glycaemia", rather than actual levels of glycaemia because there was considerable variation of basal glucose levels between animals. By displaying the data in this manner, high error terms that would have

occurred, were removed, thus showing only the true variation of glycaemia following bioassay.

3.2.5 Effects of sinus gland dilution on circulating glucose.

In order to investigate dose dependency in recipient animals after injection of crude sinus gland extract the following protocol was established. Twenty sinus glands were dissected (See Section 2.5) and homogenised in saline at 0°C. A dilution of the aliquots was then made to create a gradient of 2, 1, 0.5, 0.25, 0.1, 0.01, 0.001 and zero sinus gland equivalents in 50μl of saline. Animals were divided into eight groups, four animals in each group and a haemolymph sample obtained from each and treated as in section 2.3. Injection of the sinus gland dilutions into each animal was carried out as in section 2.3. After three hours a 50μl haemolymph sample was removed and treated as before. Circulating glucose levels were determined by the method described in section 2.4 and above in 3.2.3. Due to the delicate nature of *Nephrops*, it was considered that stress may account for part of the increase of haemolymph glucose when the animals were injected with either sinus gland extracts or reconstituted HPLC fractions. Therefore, one group of animals were handled and sham injected for one minute, the maximum period of time considered necessary for the injection of extracts or fractions.

3.2.6 Determination of CHH activity over time within *Nephrops norvegicus*.

Two groups of ten animals were used. After an initial haemolymph sample was removed, each group was recipient to 0.5 sinus gland equivalents in 50μl of saline and 50μl saline sample respectively. Haemolymph samples were then

extracted every hour for six hours and treated as in section 2.3. Bioassay for circulating glucose was then adopted as in section 2.4.

3.3 Results.

3.3.1 Sinus gland dissections.

The dissection of the sinus gland of *Nephrops norvegicus* is shown in figure 3.1. This dissection of a fresh eyestalk clearly shows the pale colouration of the gland. The sinus gland of thawed eyestalks were lighter in colour and were therefore less distinctive from the surrounding tissue thus making the dissection more difficult. In addition, collection of sinus glands from thawed eyestalks, was made more difficult by the obscuring effect that the granular pigment resulting from the ommatidia had on the preparation. Figure 3.2 demonstrates the difference between the visualisation of the dissected sinus glands of *Homarus gammarus* compared to that of *Nephrops norvegicus*.

3.3.2 Slide staining of neurosecretory material with PAF (paraldehyde fuchsin) solutions.

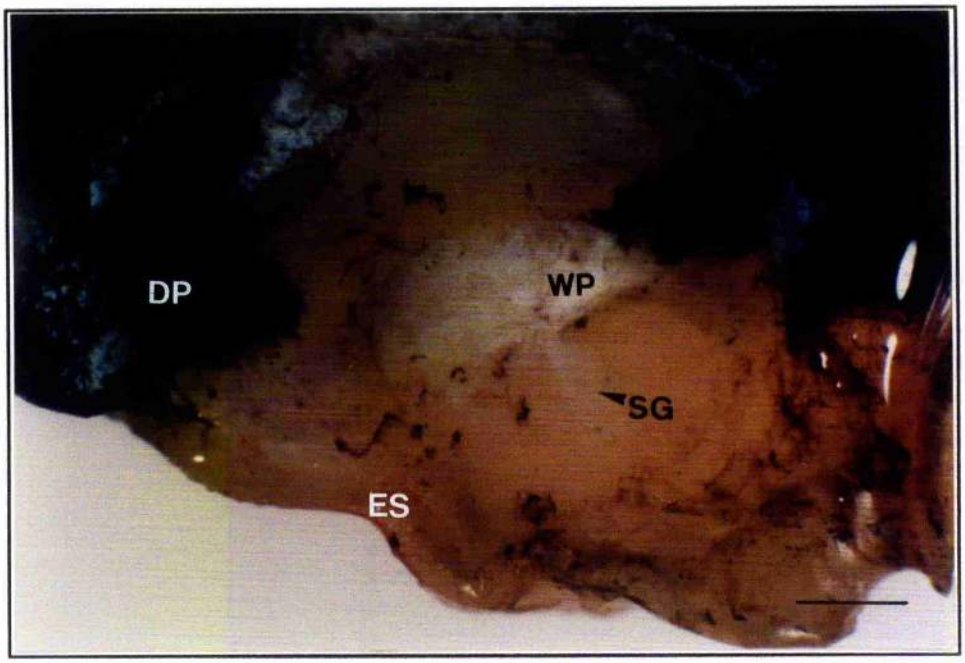
The use of the PAF staining technique enables the visualisation of neurosecretory tissue within the eyestalk, by staining specific tissues dark purple (figures 3.3 to 3.7). In figure 3.5 the sinus gland can be seen as a thickened area of cells, surrounding a central lumen, since the sinus gland is described as a neurohaemal organ, this lumen is probably a blood vessel. The axon tract is filled with neurosecretory granules and extends superficially away from the sinus gland, before descending through the neuropile to its origins as neurosecretory terminals

Figure 3.1.

Dissection of a fresh (non-frozen) eyestalk of *Nephrops norvegicus* showing the sinus gland. This figure demonstrates the difficulty of sinus gland identification from the surrounding tissue. Abbreviations: *DP* dark pigment of ommatidia; *ES* exoskeleton; *SG* sinus gland; *WP* white pigment. Scale bar = 1mm

Figure 3.2.

Dissection of the eyestalk of *Homarus gammerus* showing the detail of the sinus gland. The axon tract can be seen extending vertically away from the gland towards the X-organ (not shown). Compared to the sinus gland of *Nephrops* (figure 3.1) which is pale and diffuse, the sinus gland structure in *Homarus* can be seen as a descreate blue/white mass, with a clearly defined axon tract. The tract itself can be seen to contain neurosecretory material. At the top of the preparation is a copper grid from a transmission electron microscope, which provides a scale. Abbreviations: *AT* axon tract; *SG* sinus gland. Scale bar =250μm.


DP
WP
SG
ES

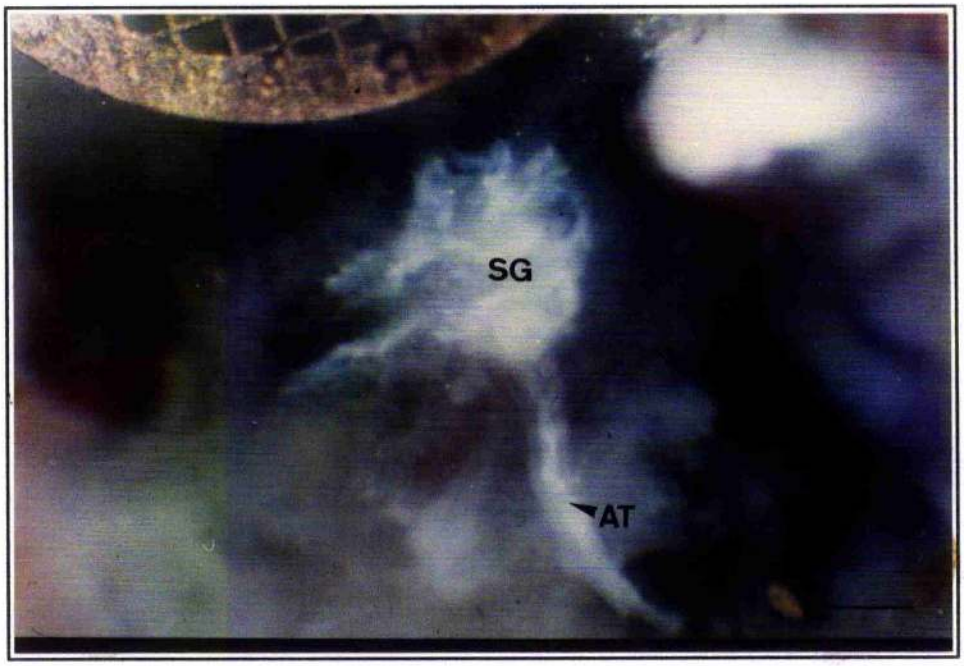
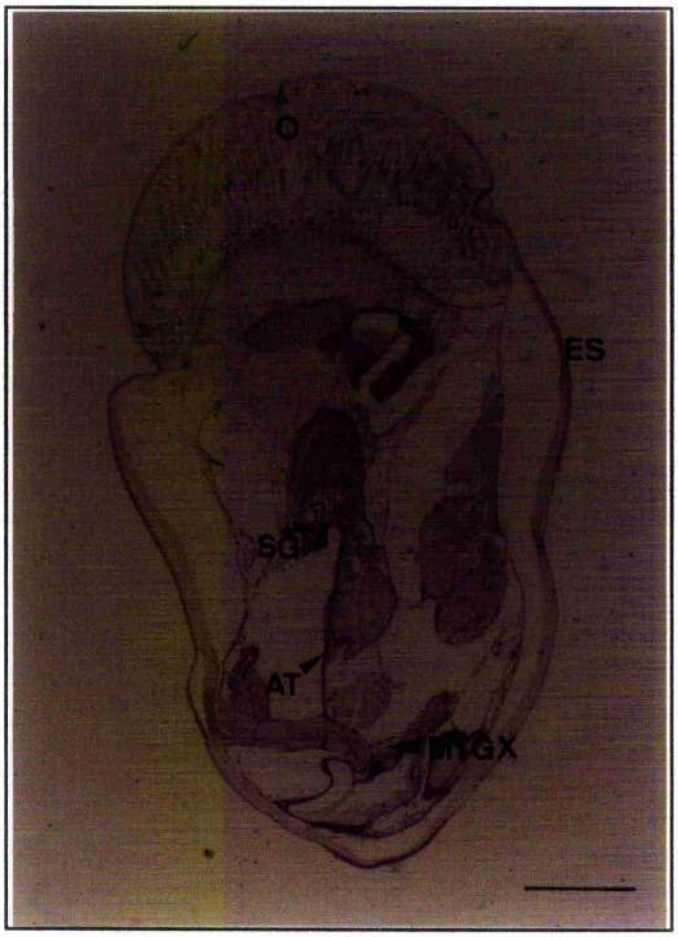

SG
AT

Figure 3.3.

Longitudinal section (8μm) of the eyestalk of *Nephrops norvegicus* stained with PAF to identify regions of neurosecretion. Neurosecretory products stain dark purple. Abbreviations: *AT* axon tract; *MTGX* medulla terminalis ganglionic X-organ; *O* ommatidia; *SG* sinus gland. Scale bar = 1mm

Figure 3.4.

Detail of longitudinal section (8μm) of the eyestalk of *Nephrops norvegicus* stained with PAF. An orange photographic filter has been used. Abbreviations: *AT* axon tract; *MTGX* medulla terminalis ganglionic X-organ; *SG* sinus gland. Scale bar = 500μm.


O
ES
SG
AT
MTGX

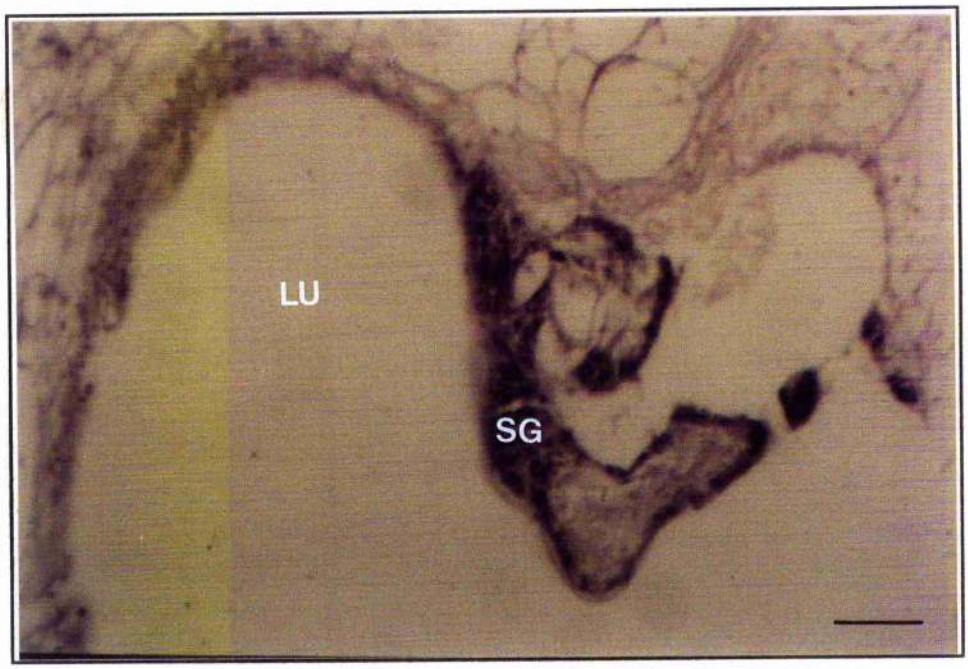

SG
AT
MTGX

Figure 3.5.

Detail of longitudinal section (8μm) of the eyestalk of *Nephrops norvegicus* stained with PAF showing high magnification of the sinus gland. Neurosecretory granules can be seen as dark purple regions. The sinus gland is surrounded by large lumens, probably blood vessels. Abbreviations: *LU* lumen; *SG* sinus gland. Scale bar = 50μm.

Figure 3.6.

Detail of longitudinal section (8μm) of the eyestalk of *Nephrops norvegicus* stained with PAF showing high magnification of the medulla terminalis ganglionic X-organ. Neurosecretory granules can be seen as dark purple regions within the cells of the perikarya. The axon tract, containing neurosecretory material, runs horizontally away from the X-organ across a block of muscle. Abbreviations: *MTGX* medulla terminalis ganglionic X-organ; *AT* axon tract. Scale bar = 50μm.


LU
SG

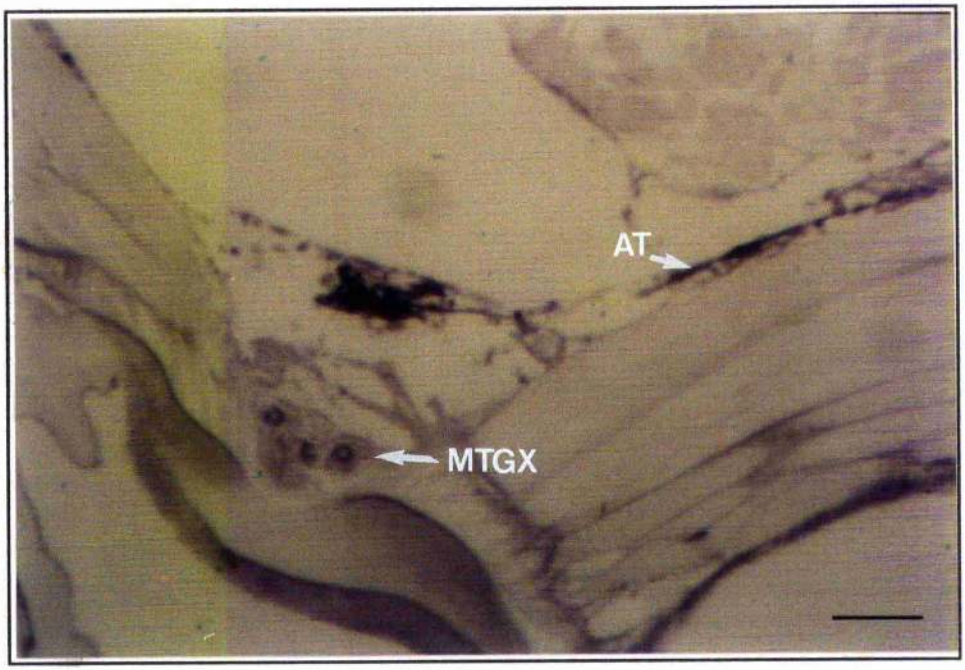
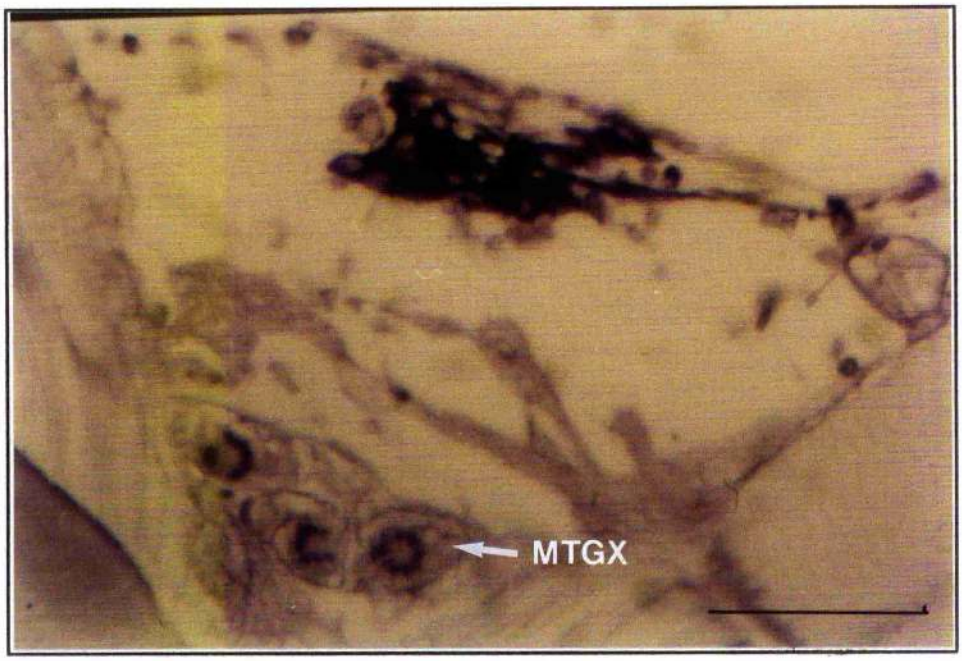

AT
MTGX

Figure 3.7.

High magnification of three individual X-organ cells containing darkly stained neurosecretory material. The dark purple area at the top of the photograph is the juxtaposition of X-organ cells and axon tract. Abbreviations: *MTGX* medulla terminalis ganglionic X-organ. Scale bar =50μm.


MTGX

adjacent to the X-organ perikarya (figures 3.6 and 3.7). These figures clearly show the occurrence of neurosecretory tissue within the X-organ perikarya.

While the staining of sectioned eyestalk with PAF produced satisfactory results, attempts to stain the XOSG complex with the dye Lucifer Yellow, proved to be unsuccessful. It was not possible to penetrate the axon tract with the micro-electrodes due to the relatively rigid nature of the tract and when penetration by the electrode did occur, the axon was punctured through the opposite wall of the tract. Bathing a preparation with the axon tract partially cut, was partially successful, although non-specific staining by the dye tended to cause the whole preparation to fluoresce under U.V. In future, the isolation of the cut axon ends with a Vaseline well or a back filling method of the axon tract, similar to that adopted by Andrew *et al* (1978), could be attempted. Gorgels-Kallen (1985) used micro injection techniques of the individual X-organ cells of *Astacus leptodactylus*, however, as the X-organ could not be observed during the dissection of *Nephrops norvegicus*, this approach would not be possible. A more effective approach may be to use immunocytochemical techniques (Dircksen, pers. comm.).

3.3.3 Calibration of both normal and micro bioassay techniques.

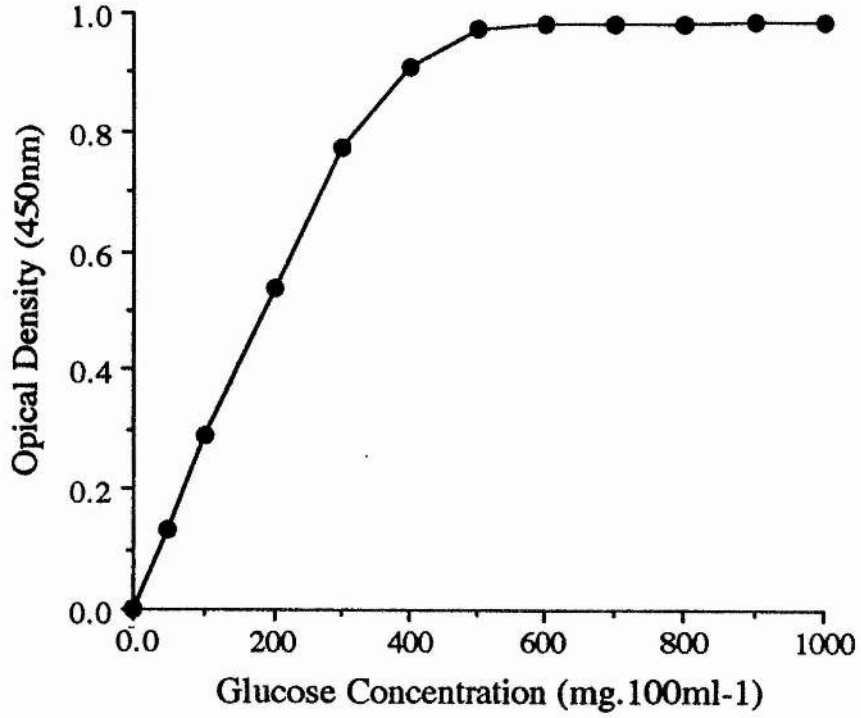
A typical calibration curve for the measurement of glucose using the standard Sigma kit is shown in figure 3.8 and demonstrates the upper limit for the assay as approximately 400mg.100ml^{-1}. As the *Nephrops norvegicus* haemolymph glucose measurements were expected to be considerably lower than this, a further calibration curve was created to measure a range of glucose from 0-100mg.100ml^{-1} and overlaid with the lower end of the original calibration curve in order to demonstrate that there was no loss of accuracy when using more dilute glucose standards (figure 3.9). As the dilution of the glucose standards was necessary in order to obtain accurate determination of haemolymph glucose, it was essential to determine that the

Figure 3.8.

Calibration curve for glucose using the standard Sigma kit protocol. The standards were measured spectrophotometrically at an optical density of 450nm. Glucose concentration is shown as mg.100ml^{-1}. For each standard n=6. Standard error bars are shown.

Figure 3.9.

Calibration curve for glucose using the standard Sigma protocol with varying concentration of standards. Closed circles indicate concentrations of glucose of 0, 50 and 100 mg.100ml^{-1} from figure 3.6. Open circles represent a separate calibration curve, glucose concentrations from 0-100mg.100ml-1, thus ensuring accuracy with dilution. All standards were measured spectrophotometrically at an optical density of 450nm, (n=6). Standard error bars are shown.


1.0
0.8
0.6
0.4
0.2
0.0
Opical Density (450nm)
0.0
200
400
600
800
1000
Glucose Concentration (mg.100ml-1)

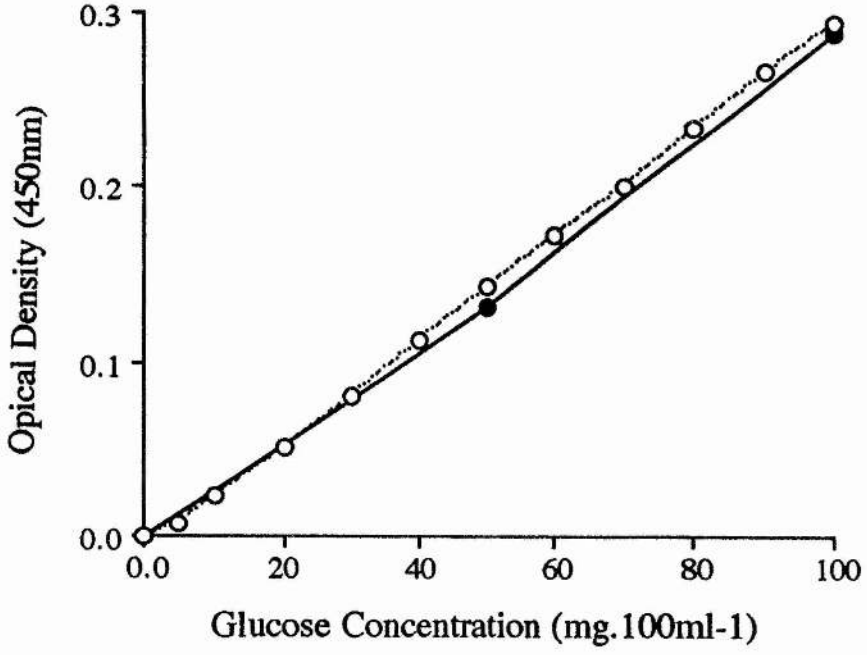
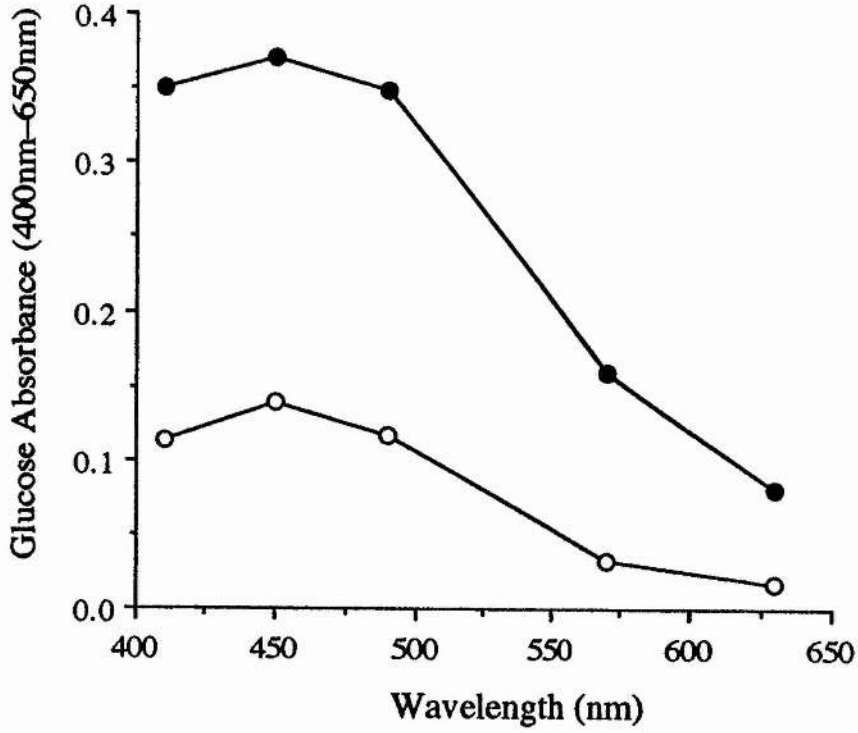

0.3
0.2
0.1
0.0
Opical Density (450nm)
0.0
20
40
60
80
100
Glucose Concentration (mg.100ml-1)

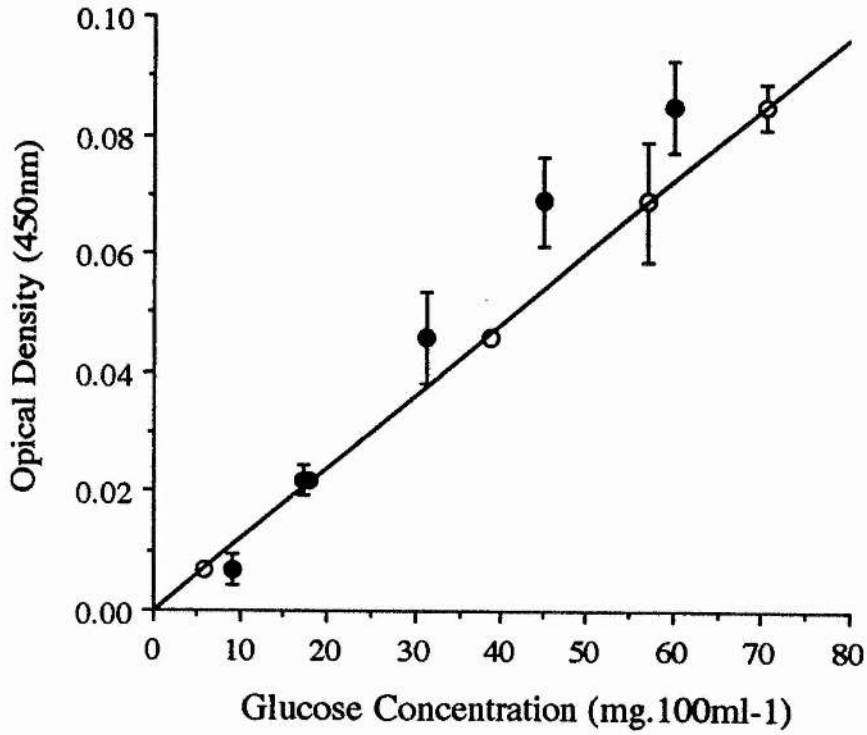
Figure 3.10.

The optical density of the enzymatic reaction is measured over varying wavelength to determine if 450nm is optimal for absorption . Closed circles show a 50μl glucose standard of 10mg.100ml^{-1} and open circles show a 10μl sample of 10mg.100ml^{-1} (n=6). Standard error bars are shown.

Figure 3.11.

The comparison between the calibration curve for glucose using the standard Sigma protocol incubated normally and shown as open circles and the glucose bioassay, displayed as closed circles, which was incubated in the micro plate using a standard of 10mg.100ml^{-1} (n=6). See chapter 2, section 2.4.


Glucose Absorbance (400nm–650nm)
0.4
0.3
0.2
0.1
0.0
400
450
500
550
600
650
Wavelength (nm)


Opical Density (450nm)
0.10
0.08
0.06
0.04
0.02
0.00
0
10
20
30
40
50
60
70
80
Glucose Concentration (mg.100ml-1)

optical density of the enzymatic reaction was still optimal at 450nm. These data are displayed as figure 3.10.

As previously described in Chapter 2 and Chapter 3, it became necessary to produce a micro assay of the Sigma kit. The following figures describe the data obtained in order to demonstrate that the normal Sigma assay and the micro assay which was developed for this work were compatible. The normal assay, when carried within the micro plate wells, overloads above 40mg.100ml^{-1} necessitating the further dilution of the glucose standards for a within well reaction. In order that this latter method could be verified as an acceptable bioassay for the plate incubated determination of haemolymph glucose, both the plate incubated assay and the normal assay were compared by superimposing the two calibration curves. Preliminary measurements of the circulating haemolymph glucose levels in *Nephrops norvegicus*, both before and after sinus gland extracts were injected, indicated that values in excess of 60mg.100ml^{-1} would not be obtained. The juxtaposition of both plate incubated assay and normal bioassay standard curves are shown in figure 3.11. Similar results of hyperglycaemia in *Nephrops* have been demonstrated by Leuven *et al* (1982).

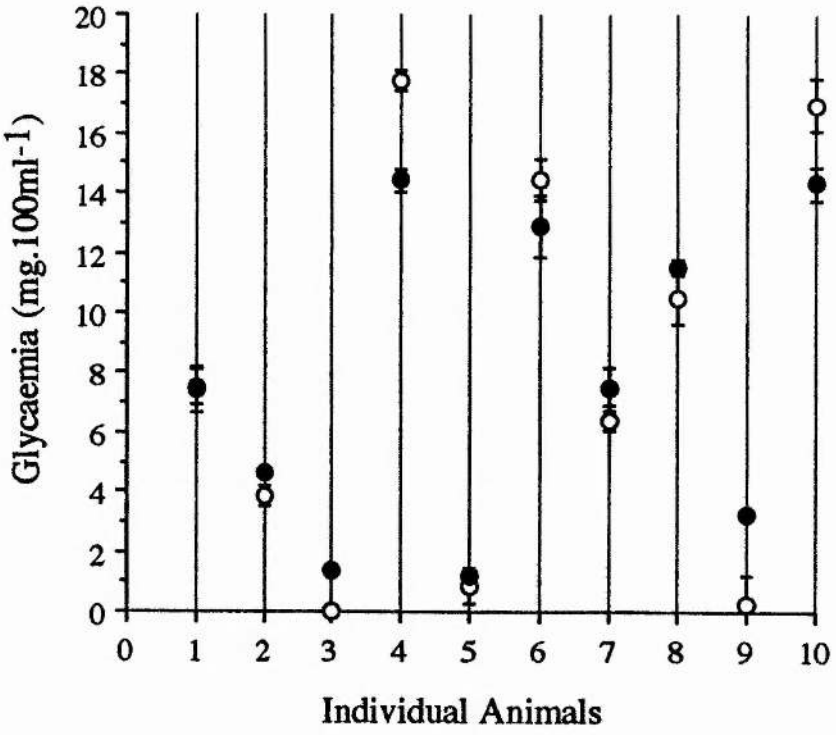
Ultimately, it was necessary to demonstrate that the two bioassay methods were comparable on actual haemolymph glucose measurements. Figure 3.12 indicates the basal haemolymph glucose levels of ten animals (measured in triplicate) and demonstrates the differences between the two techniques. Additionally, this figure highlights the point made earlier, that it proved necessary to display glycaemia graphically as "Change of Glycaemia" due to the variation of basal glucose levels between animals. The data are presented as mean ± SD in figure 3.13

Figure 3.12.

The measurement of basal haemolymph glucose (mg.100ml^{-1}) in *Nephrops norvegicus*. Open circles show the haemolymph glucose concentration obtained using the standard Sigma glucose determination protocol, while the closed circles show the value obtained using the plate incubated bioassay described in chapter 2, section 2.4. For each value, n=4. Inter-individual variation of haemolymph glycaemia in *N. norvegicus* is additional demonstrated. Vertical lines enable easy identification of individual animals.

Figure 3.13.

The means and ± sem of haemolymph glucose (mg.100ml^{-1}) of *Nephrops norvegicus* determined using the standard Sigma bioassay and the plate incubated method described in chapter 2, section 2.4 are shown. (n=10). A non-significant difference was determined by means of the bioassays using the Student *t*-test.


Glycaemia (mg.100ml-1)
20
18
16
14
12
10
8
6
4
2
0
0 1 2 3 4 5 6 7 8 9 10
Individual Animals

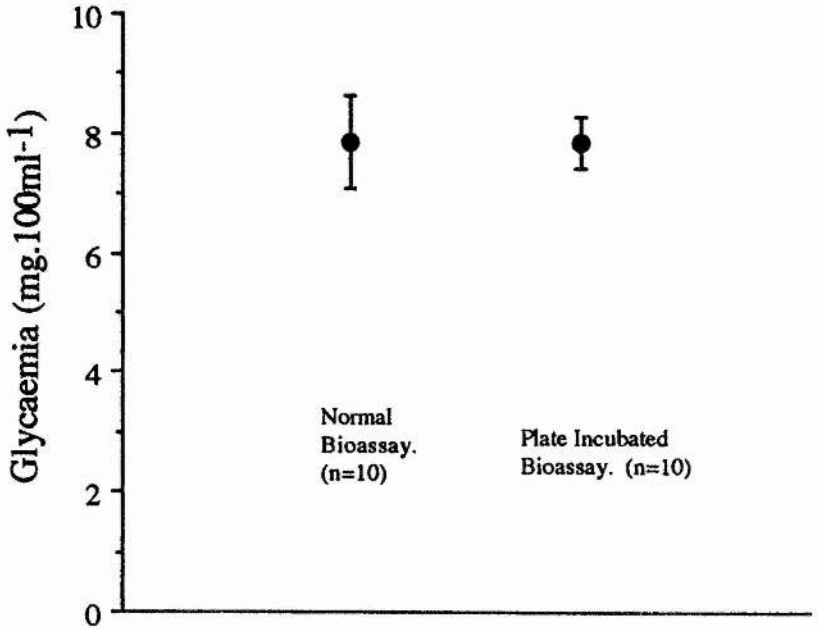
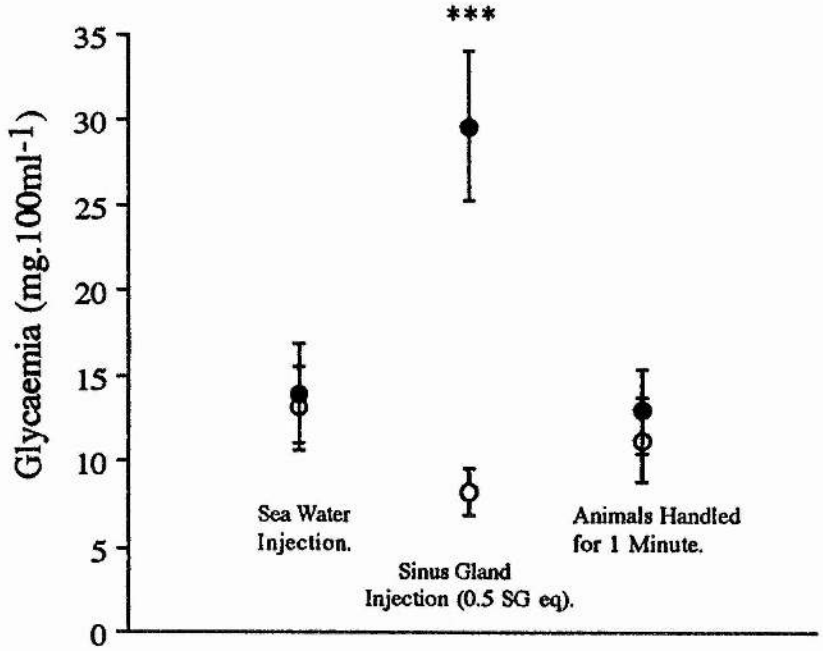

Glycaemia (mg.100ml-1)
10
8
6
4
2
0
Normal Bioassay. (n=10)
Plate Incubated Bioassay. (n=10)

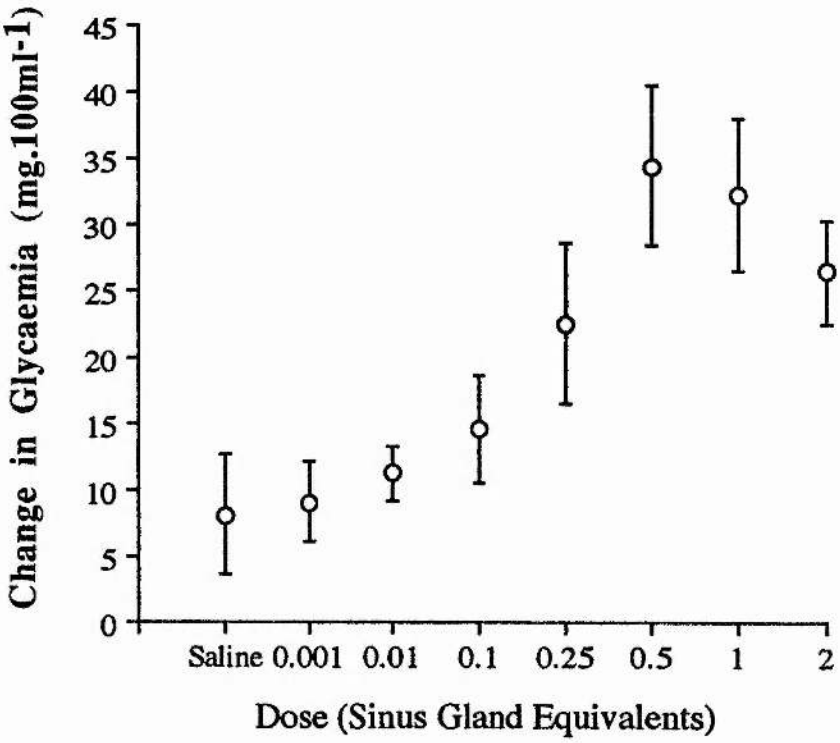
Figure 3.14.

Variations of glycaemia in *Nephrops norvegicus* (mg.100ml^{-1}) following three treatments; saline injection, 0.5 sinus gland equivalents and handling for 1 minute. For each treatment mean values ± sem (n=10). Open circles indicate glucose concentration prior to treatment and closed circles show glucose concentration after treatment. Saline and sinus gland treatments had haemolymph glucose determined three hours following injection of sample. Only an injection of 0.5 sinus gland equivalents demonstrated a significant increase of glycaemia (Student *t*-test = $p<0.001$).

Figure 3.15.

Dose response effects of sinus gland extract injections on induced hyperglycaemia in *Nephrops norvegicus* in mg.100ml^{-1}. Each value is the mean ± sem of n=8 individuals with differences of haemolymph glucose concentration determined 3 hours following injection of sinus gland extract. A control injection consisted of twice filtered sterile sea water (TFSSW).


***
Glycaemia (mg.100ml-1)
35
30
25
20
15
10
5
0
Sea Water Injection.
Sinus Gland Injection (0.5 SG eq).
Animals Handled for 1 Minute.


Change in Glycaemia (mg.100ml-1)
45
40
35
30
25
20
15
10
5
0
Saline 0.001 0.01 0.1 0.25 0.5 1 2
Dose (Sinus Gland Equivalents)

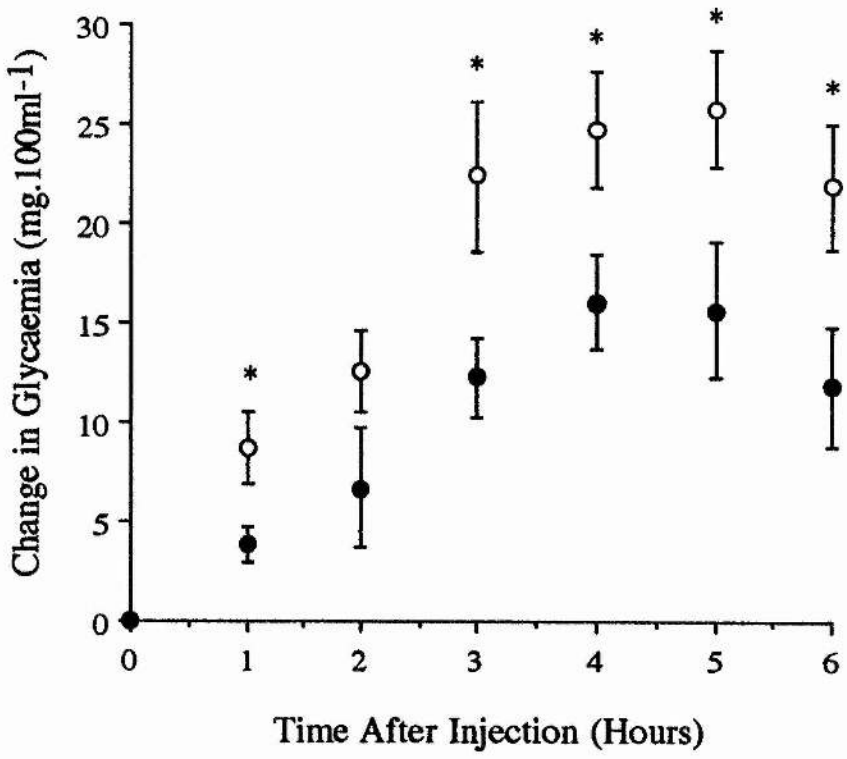
3.3.4 Effect of injection of crude sinus gland extracts on glycaemia in *Nephrops norvegicus*

Preliminary experiments determined the parameters of the response of *Nephrops norvegicus* to injections of crude sinus gland extract. Figure 3.14 demonstrates the difference in haemolymph glucose between animals injected with twice filtered sterile sea water and 0.5 sinus gland equivalents. The effect of handling for one minute and sham injection on the levels of glycaemia are also displayed. The hyperglycaemic effect of a dilution gradient of crude sinus gland injections into *Nephrops* is shown in figure 3.15. The results demonstrate that a significant increase in haemolymph glucose is obtained following an injection of 0.25 sinus gland equivalents. Injections of larger amounts of crude sinus gland extract resulted in maximum hyperglycaemia with 0.5 sinus gland equivalents, while 2 sinus gland equivalents showed a significant inhibition of hyperglycaemia. From these data it is proposed that HPLC fractions and all positive controls should be tested at a dosage of 0.5 sinus gland equivalents.

Injection of crude sinus gland extract into *Nephrops norvegicus* resulted in an increase in haemolymph glucose which was apparent 1h after injection. (Figure 3.16). Hyperglycaemia induced by 0.5 sinus gland equivalents reached a maximum value after five hours and then began to decline. It should also be noted, however, that an increase was also observed in individuals that had been injected with saline which may be attributable to stress caused by repeated injections. It was commonly observed in experiments that there was considerable inter-individual variation in baseline haemolymph glucose, and in the response of the individuals to sinus gland injection. The net change in glucose between animals was similar, however, and for this reason data are represented as change in glycaemia. The greatest significant difference between the test and the control animals was observed after six hours and the most significant increase of glycaemia was obtained after five hours. However,

Figure 3.16.

Change in haemolymph glucose ($mg.100ml^{-1}$) following injection of a) crude extract of sinus gland (0.5 sinus gland equivalents) (open circles) or b) isotonic saline (closed circles). Data shown are means ± sem (n=5) for glucose at hourly intervals up to 6 hours post-injection. Significant differences compared to the TFSSW injected animals were determined by means of the Students *t*-test and are indicated by asterisks: * = $p<0.05$


Change in Glycaemia (mg.100ml-1)
30
25
20
15
10
5
0
0
1
2
3
4
5
6
Time After Injection (Hours)
*
*
*
*
*

the increase in circulating glucose after three hours was sufficient to observe a difference between the test and control animals and allowed realistic bioassay times. Therefore, bioassay of HPLC fractions was carried out with a three hour sampling time between injection of sample and the assessment of haemolymph glucose.

3.4 Discussion

Hyperglycaemic responses to the injection of crude eyestalk extracts have been shown in a number of species but the time of maximal glycaemia is seen to be variable between species. Injection of crude sinus gland extracts into juvenile *Homarus americanus* (Soyez *et al*, 1990) has shown a maximal hyperglycaemic effect after three hours, while the hyperglycaemic effect in the crayfish *Orconectes limosus* is maximal after two hours (Keller and Andrew, 1973). *Nephrops norvegicus* initially shows a similar pattern to that of *H. americanus*, however, glucose levels continue to rise until five hours after the injection of the sinus gland extracts. The maximum increase of haemolymph glucose in *N. norvegicus* is rather low (about 30mg.100ml^{-1}) when compared to values obtained from crayfish, (80-100mg.100ml^{-1}) (Soyez *et al*, 1990).

Results obtained by Leuven *et al* (1982), however, demonstrated considerably higher levels of hyperglycaemia in *Nephrops norvegicus* when injected with eyestalk extracts from *N. norvegicus* and *Astacus leptodactylus* and even saline than in this particular study. In addition, haemolymph samples were obtained from *N. norvegicus* after only two hours, a period of time which has been demonstrated by this present work, to be within the maximal hyperglycaemia effect. Feeding is known to affect glycaemia (Parvathy, 1971; Chan *et al*, 1988; Keller and Orth, 1990) by increasing haemolymph glucose, although during starvation the levels of CHH also rise (Keller and Orth, 1990). The production of CHH has been shown to stimulate amylase secretion from the midgut of *Orconectes limosus* (Sedlmeier,

1988), however, amylase secretion increases during starvation (Fingerman *et al*, 1967). This may be the result of CHH release due to the starvation stress. A similar fluctuation of circulating glucose occurs depending on the reproductive condition of the animal (Dean and Vernberg, 1965a). In the crab, *Callinectes sapidus*, for example, glucose levels were similar between an immature female and a male. However, when the female was in an early ovigerous stage, an increase of between two and three times in the circulating glucose levels were obtained. Similar increases were observed in *Hepatus ephelitus*, *Panopeus herbstii* and *Libinia emarginata*. Fluctuations in circulating glucose occur at various stages of the crustacean moult cycle (Telford, 1968; Gwinn and Stevenson, 1973; Chan *et al*, 1988). Increases of circulating glucose were considerably greater in premoult *Homarus americanus*, than during the intermoult period, while postmoult animals displayed a reduced level of haemolymph glucose (Telford, 1968). Differences in the feeding behaviour of crustaceans during the moult cycle are thought to provide an explanation for this variation (Chan *et al*, 1988), although, chitin absorption prior to moult may explain the high levels of hyperglycaemia (Gwinn and Stevenson, 1973). Circulating glucose has been demonstrated to be associated with day length, with fluctuations shown to correspond to fixed periods of light or dark (Dean and Vernberg, 1965a; Hamann, 1974; Gorgels-Kallen, 1985; Kallen *et al*, 1988; Kallen *et al*, 1990, Keller and Orth, 1990; Tensen, 1991). This and other physiological responses to altered environmental conditions are discussed in more detail in chapters 1 and 5. These effects have all been minimised in this study (see Chapter 2).

The maintenance of the water temperature in which the *Nephrops norvegicus* were stored, varied throughout the year, since the water that is in the St. Andrews aquarium is taken directly from the sea. Since temperature noticeably increases haemolymph glucose levels in crustaceans (Dean and Vernberg, 1965b; Keller and Orth, 1990) and also circulating levels of CHH (Keller and Orth, 1990), it is

possible that this may account, in part, for the differences in levels of haemolymph glucose. The effects of temperature have only been shown to effect resting glucose levels, and have not shown an increase on top of that which normally occurs following sinus gland injection. It is considered that a lower temperature will reduce the animals energy requirments to a minimal level (Dean and Vernberg, 1965b), therefore, a lower average sea temperature at St. Andrews compared to the 13°C at which *N. norvegicus* were maintained in the Leuven *et al* (1982) study may explain a lower hyperglycaemia level due to a lower energy requirement. The depth at which *N. norvegicus* are caught and hence the temperature at which they are naturally maintained may in addition effect hyperglycaemia. If *N. norvegicus* are kept at temperatures above that of the natural environment, they may experience a higher energy requirement, thus giving rise to greater hyperglycaemic fluctuations. Lower aquarium temperatures may have the opposite effect. For details of temperature effects see Chapter 1.

Glucose represents only 50-60% of circulating sugars in lobster haemolymph (Telford, 1965), therefore, it should be noted that the bioassay used in this study, being glucose specific, does not take account of possible changes in the levels of other sugars such as fructose, galactose or maltose. Levels of these and other sugars have been investigated in some crustaceans (Telford, 1968). Although effects of sinus gland extract injection on other sugars is not well documented, Soyez *et al* (1990) showed no increases of the sugar trehalose.

The low level of hyperglycaemic response in *Nephrops norvegicus* make this species more difficult to investigate and less amenable to bioassay than some other crustacean species. Low hyperglycaemic responses to crude sinus gland extract and HPLC fractions have a tendency to be masked by stress and/or diurnal fluctuations in circulating glucose (see Chapter 5), in addition to inter-individual variation in basal glucose levels. The role of the heterologous bioassay animal, in this study *Pacifastacus leniusculus*, becomes an important one. The use of a heterologous

bioassay was demonstrated by Leuven *et al* (1982) using *Astacus leptodactylus* for assay of CHH from a number of species. The value of the *P. leniusculus* heterologous bioassay in this study has proved to be the more sensitive measurement of hyperglycaemia-inducing properties of partially purified extracts from *Nephrops*. Levels of glycaemia induced in *P. leniusculus* were comparable to those obtained in *Orconectes limosus* (Keller and Orth, 1990) and although the upper limit of the hyperglycaemic response often exceeded the 60mg.100ml^{-1} level, which was considered to be accurate for the micro assay method, the heterologous bioassay was intended only as a marker for active and non-active fractions. Ultimately, all activity has been assessed in *N. norvegicus* itself.

Finally, the effect of handling stress hyperglycaemia (Riegel, 1960; Telford, 1968), may effect any future results. A normal bioassay requires two injections, three hours apart, each lasting for under one minute. Figure 3.14 demonstrates that one minute of handling and sham injection had little effect on glycaemia and should therefore not effect simple bioassay results. In figure 3.16, when animals are injected with saline and sinus gland extracts and repeatedly sampled each hour, stress hyperglycaemia may affect results. It is possible therefore, that the extension of hyperglycaemia for up to five hours post sinus gland injection, may be the result of repeated sampling and thus stress effects maintain artificially high levels of haemolymph glucose.

Chapter 4.

Purification, isolation and characterisation of crustacean hyperglycaemic hormone from *Nephrops norvegicus*.

4.1 Introduction.

In recent years, the use of high performance liquid chromatography (HPLC) has proven to be a powerful tool for the isolation of neuropeptides from the sinus gland of crustaceans. The isolation, purification and chemical characterisation of CHH has resulted in the authentication of a distinct peptide family that exists not only within a single species, but also extends across species boundaries. Keller (1977; 1981), Keller and Wunderer (1978) and Keller *et al* (1985) demonstrated molecular polymorphism between different species, while Skorkowski *et al* (1977), Martin *et al* (1984) and Van Wormhoudt *et al* (1984) identified polymorphism within the same species. More recent work using HPLC separation of sinus glands confirmed the occurrence of molecular polymorphism of CHH within the same species (Newcomb, 1983; Stuenkel, 1983; Keller *et al*, 1985; Newcomb *et al*, 1985; Huberman and Aguilar, 1986; Huberman and Aguilar, 1988). In *Homarus americanus*, up to four fractions with hyperglycaemic activity have been identified (Soyez *et al*, 1990; Tensen *et al*, 1989) and the structural basis for these isoforms has recently been elucidated for two *Homarus* CHH molecules; *Hoa* CHH A and *Hoa* CHH B (Tensen *et al*, 1991c). The structural basis of these isoforms is discussed in Chapter 1 and a review of this peptide group is discussed by Keller (1992).

The occurrence of polymorphism has been well documented in both invertebrates and vertebrates (Lynch and Snyder, 1986) and is the result of either sequence heterogeneity or partial post-translational modifications of L amino acid residues into D amino acid residues. The latter explanation has been most strongly postulated for the polymorphism that occurs in CHH and GIH (Tensen, 1991). This may alter the secondary structure within the N-terminus of the peptide which in turn may effect the elution patterns obtained from the HPLC. However, they have similar physicochemical characteristics (see Chapter 1).

The molecular mass of isoforms of *Hoa* CHH have been shown to be similar (Soyez *et al*, 1990) and the same authors suggest isoform differences may lie in the positioning of disulphide bridges. In addition the L and D form may show affinities for different types of receptor, which may explain variations of hyperglycaemic responses obtained during heterologous bioassay (Tensen *et al* 1989, Tensen, 1991). This suggests that some CHH isoforms are only active at the species level and not, as previously described, at the infra-order level (Leuven *et al*, 1982).

This chapter describes the isolation and purification of CHH from the sinus glands of *Nephrops norvegicus* using HPLC, and determines the extent of CHH polymorphism in this species. The process of isolation was monitored by ELISA using a polyclonal antibody against *Orconectes* CHH. Additionally, the hyperglycaemic activity was assessed using the bioassay discussed in Chapter 3, while the purified peptides were characterised by the determination of their molecular weights using SDS-PAGE electrophoresis. The purity of HPLC fractions, prior to amino acid analysis, was determined using capillary electrophoresis (CE).

4.2 Materials and methods.

4.2.1 Maintenance of animals and collection of sinus glands.

Specimens of the Norway Lobster, *Nephrops norvegicus*, were obtained from the Forth/Cromarty region of the North Sea and maintained in an aquarium in conditions described in Chapter 2. Due to the high level of mortality observed in this species when held in aquaria and considering its high market price, it was pertinent to ablate the eyestalks of any dead or dying animal, in order that these may be stored and used for sinus gland dissection at a later date. This was only

practised within the first 12 hours following the landing of the animals by the fisherman. The majority of the eyestalks from *N. norvegicus*, were obtained from animals caught on the west coast of Scotland. Without taking into account either, sex or the moulting stage of the animals, the eyestalks, when ablated, were frozen on site in liquid nitrogen or on solid CO_2, and lyophilised at the laboratory and stored under nitrogen or helium. Fresh sinus glands were obtained from freshly sacrificed animals taken from the laboratory aquarium. The preparation of eyestalk neuropeptides by both the hot HCl method or the cold acetic acid method, yielded similar results and are described in detail in Chapter 2. Occasionally, eyestalks of *Homarus gammarus* were collected in order to use the sinus glands from this species, as a positive anti-GIH control during the ELISA (see Chapter 2 and later in this Chapter).

4.2.2 Sep Pak C18 cartridge purification of sinus gland extract.

Prior to the fractionation of sinus gland extracts on the HPLC systems, prepared extracts were applied to a C18 Sep Pak cartridge (Waters) in order to determine the percentage of solvent B (acetonitrile/0.1% TFA) would elute the majority of hyperglycaemic factors. This was not used as an initial purification step, but as an indication of the gradient of solvent that would be required for the HPLC purification of the sinus gland extracts. The Sep Pak cartridge was prepared for use by the application of 5ml of 100% methanol, followed by 10–20ml of MilliQ water to remove any traces of the methanol. Once the column was prepared, a lyophilised extract containing 10 sinus glands was resuspended in 1ml of MilliQ water, and passed slowly through the Sep Pak using a 1ml syringe barrel. Once loaded with extract, the cartridge was washed, first with 5mls of MilliQ water, followed by 5mls of each of 25%, 50% and 100% acetonitrile. Each of the fractions that was collected was lyophilised and

reconstituted in 1ml of MilliQ water and a hyperglycaemic bioassay was carried out as described in Chapters 2 and 3.

4.2.3 HPLC purification of sinus gland extracts.

The HPLC of prepared sinus gland extracts was carried out as described in Chapter 2. However, a second HPLC step was performed following each of the initial HPLC separations. This second step was identical to acetonitrile method described in Chapter 2, except that the gradient, over which the fractions of interest were obtained, was extended. The details of the gradients adopted for each of the HPLC methods are described in the figure legends.

4.2.4 Determination of immunopositive fractions from HPLC using ELISA.

For the immunochemical determination of *Nephrops norvegicus* CHH and GIH material from HPLC fractionation, polyclonal antisera raised against CHH of the crayfish, *Orconectes limosus*, and GIH of the lobster, *Homarus americanus*, were used. Details of these antisera are described in Keller *et al*, (1985) and Meusy and Soyez, (1991), respectively. Identification of immunoactive CHH or GIH material in the eluted HPLC fractions was performed by direct ELISA, the details of which, are described in Chapter 2.

Lyophilised HPLC fractions were reconstituted in their original volume of PBS (0.75ml or 1.0ml). 0.5 sinus gland equivalents of each fraction were pipetted in triplicate into each well and a direct ELISA was performed in microtitre plates by the method discussed previously in Chapter 2. Immunopositive fractions were then bioassayed for hyperglycaemic activity.

In order that a degree of calibration of antigen to antibody could be determined, a dilution gradient was created for the fraction with both the greatest CHH immunoactivity and hyperglycaemic activity. A known dilution of sinus gland equivalents was constructed and using these, a direct ELISA was carried out as described previously.

4.2.5 Bioassay of immunoactive HPLC fractions.

The CHH bioassay was carried out as described in Chapter 2. Details of the experimental design are described below.

Once the HPLC fractions had been tested using the anti-*Orconectes* CHH antisera, the peaks of immunoactivity were bioassayed in order to test for *in vitro* CHH activity in the host animal. Animals were split into four groups of five animals:

Group 1 negative control animals injected with 50μl of saline,

Group 2 positive control animals injected with 50μl of saline containing 0.5 sinus gland equivalents,

Group 3 stress control animals, where animals had haemolymph removed only at the start and finish of the experiment in order to monitor any handling stress within the control animals,

Group 4 test animals injected with 50μl (0.5 sinus gland equivalents) of immunopositive fraction.

All experiments were carried out in an identical manner described above and in Chapter 2, however, the animals that were injected with immunopositive fractions were injected with increasing concentrations of sinus gland equivalents.

4.2.6 Protein and molecular weight determination of HPLC fractions.

The protein content of purified fractions was determined by method described in Chapter 2 (Smith *et al*, 1985). Finally, a modification of the discontinuous SDS-PAGE procedure used by Schägger and von Jagow (1987) was adopted to estimate the molecular weight of peptides separated using the HPLC system described in Chapter 2. Prior to amino acid analysis of the CHH active fractions, 15 sinus gland equivalents of the relevant fraction was applied to the CE (Bio Rad) to enable the accurate purity of the fraction to be determined (see Chapter 2 for details). Wavelengths between 200nm and 300nm were continuously monitored, using the multi-wavelength facility incorporated within the BioFocus 3000.

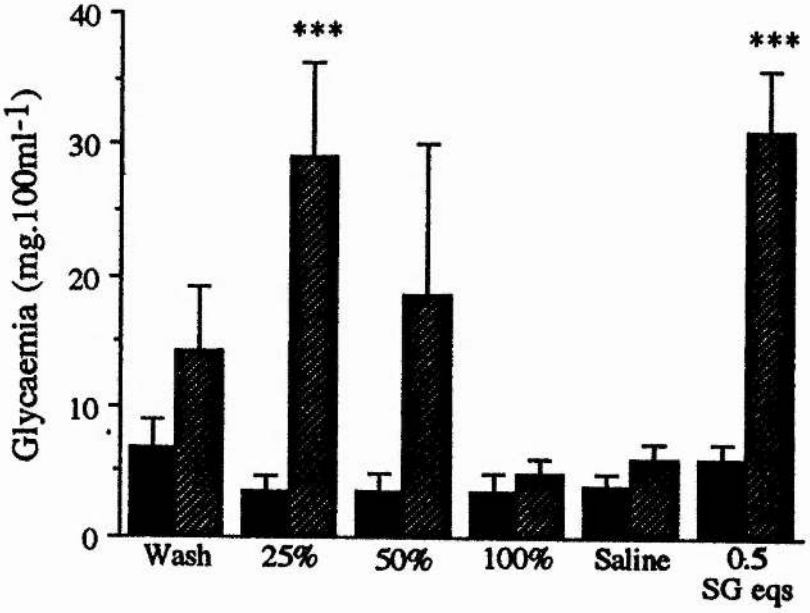
4.3 Results.

4.3.1 Purification of sinus gland extract using Sep Pak C18 cartridges.

The hyperglycaemic activity of crude sinus gland extracts from *Nephrops norvegicus*, that had been extracted using 2M acetic acid, is shown in figure 4.1. The results demonstrate that a significant increase of haemolymph glucose is obtained following elution of hyperglycaemic peptides with 25% acetonitrile, though there is still notable hyperglycaemic effect with the 50% acetonitrile eluent. It appears that 100% binding of hyperglycaemic factors does not occur using this type of reverse phase support, as the wash of the loaded cartridge with MilliQ water retains a small hyperglycaemic capability. It is clear, however, that 50% acetonitrile removes virtually all the hyperglycaemic propensity of the sinus gland extract, as the 100% wash displays a non-significant increase of glucose.

Figure 4.1.

Hyperglycaemic activity of crude sinus glands extracted using 2M acetic acid, applied to a C18 Sep Pak cartridge (Waters) and elluted with water, 25% acetonitrile, 50% acetonitrile and 100% acetonitrile. Each fraction was lyophilised, reconstituted in 1ml of MilliQ water and 0.5 sinus gland equivalents of each was injected into *Nephrops norvegicus* (n=6). Positive controls (0.5 crude sinus gland extract) and negative controls (TFSSW) are shown on the right of the figure. Solid bars indicate initial mean haemolymph glucose levels and the hatched bars show mean haemolymph glucose levels 3 hours following injection of treatment. All values show actual glycaemia in $mg.100ml^{-1}$. Significant differences compared to the initial glucose concentrations were determined by means of the Students *t*-test and are indicated by asterisks: * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.


Glycaemia (mg.100ml-1)
40
30
20
10
0
***
***
Wash
25%
50%
100%
Saline
0.5
SG eqs

It is noteworthy however, that pretreatment of samples by C18 Sep Pak cartridges have been shown to bind some peptides irreversibly to this type of support medium, ultimately resulting in greater losses of some peptides than others (Keller and Kegel, 1984).

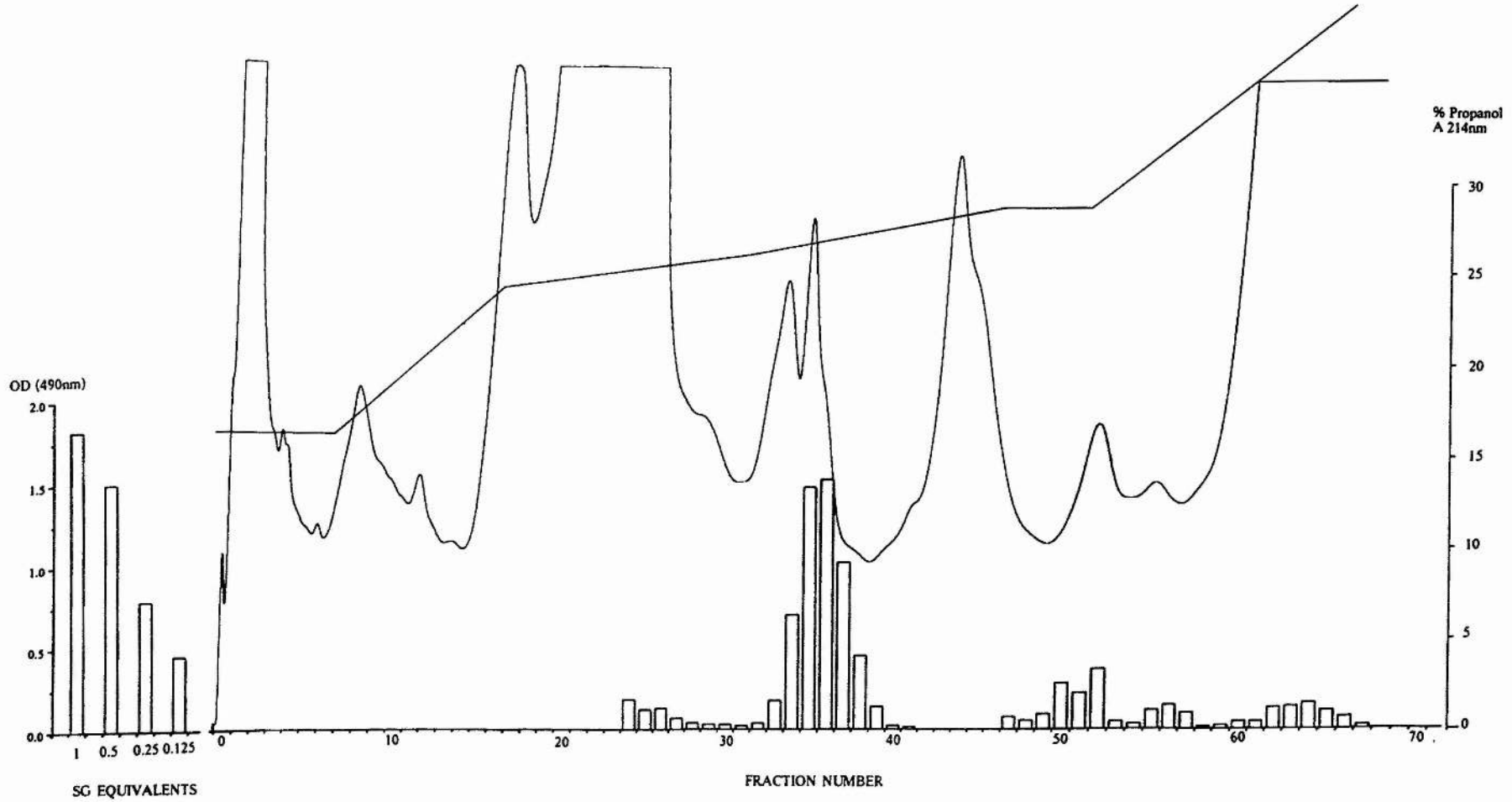
4.3.2 RP HPLC analysis of *Nephrops norvegicus* sinus gland extracts.

A typical chromatogram of 130 sinus glands separated on C18 reverse phase HPLC is shown in figure 4.2. Hyperglycaemic immunoreactivity, as identified using the anti-*Orconectes* CHH antiserum in the ELISA and shown as a superimposed bar graph in figure 4.2, is associated mainly with a single peak (fractions 35+36) eluting at approximately 23% propanol, however, other lesser areas of immunoactivity are also observed which correspond to smaller, less well defined peaks on the chromatogram. The immunoreaction of a dilution series of crude sinus gland extract, is depicted on the left side of the figure. Although the extent of polymorphism of CHH, that has been demonstrated in other decapod species does not appear to exist in this species, the peak associated with fractions 35 and 36 may be comprised of two closely eluting peaks. Rechromatography of these fractions using a reverse phase column with an acetonitrile gradient, produced a profile consisting of numerous small peaks with little or no immunoactivity.

A typical chromatogram of an extract of 300 sinus glands from *Nephrops norvegicus*, separated using a reverse phase column with acetonitrile method is shown in figure 4.3. CHH and GIH immunoreactivity was determined for each fraction by means of the ELISA detection method described in Chapter 2. These results are displayed by means of the superimposed bar graph in figure 4.3.1. The CHH immunoactivity is associated with a region extending from fractions

Figure 4.2.

Chromatogram obtained by gradient separation reverse phase HPLC of extract of 130 sinus glands on a Capital Nucleosil C-18 column (5µm particle size, 250mm x 46mm internal diameter). Mobile phases were 0.1% TFA (solvent A) and 0.1%TFA in 1-propanol (Solvent B) with a gradient (indicated by a solid line) from 16% B to 50% B and a flow rate of 0.75 $ml.min^{-1}$. U.V. absorbance was monitored at 214nm and fractions were collected at 1 minute intervals. ELISA using the polyclonal rabbit anti-*Orconectes* CHH antisera, was performed on collected fractions and resulting optical density recorded at 490nm is shown as a histogram with maximal absorbance (immunopositive reaction) being associated with fractions 35 and 36. Details of the ELISA protocol and antisera used are described in the text. Optical density of crude sinus gland extracts (following ELISA) are shown to the left of the figure and indicate relative levels of activity.


OD (490nm)
2.0
1.5
1.0
0.5
0.0
1
0.5
0.25
0.125
SG EQUIVALENTS
% Propanol
A 214nm
30
25
20
15
10
5
0
0
10
20
30
40
50
60
70
FRACTION NUMBER

39–60, eluting at approximately 50% acetonitrile. However, the greatest activity is associated with three separate peaks. The GIH however, shows immunoactivity with two separate areas; firstly with fractions 41–46, the same area that is immunoactive for CHH, and secondly, with an area extending from fraction 50–56, eluting at approximately 70% acetonitrile. This second zone of activity appears to be associated with two peaks.

Following the assessment of the peak purity and an estimation of molecular weight by SDS-PAGE (figures 4.5 and 4.6) (see section 4.3.3), fractions 39–45 were lyophilised, reconstituted in 175μl and reapplied to the column, using the extended gradient described earlier in an attempt to separate the peaks. The chromatogram of this second HPLC purification step is shown in figure 4.7 and the associated CHH immunoactivity is displayed as a superimposed bar graph as figure 4.7.1. It appears that the CHH immunoactivity is associated with each of the fractions containing peak (I) and peak (II), however, peak (III) appears to have little immunoactivity.

4.3.3 Molecular weight determination by SDS-PAGE.

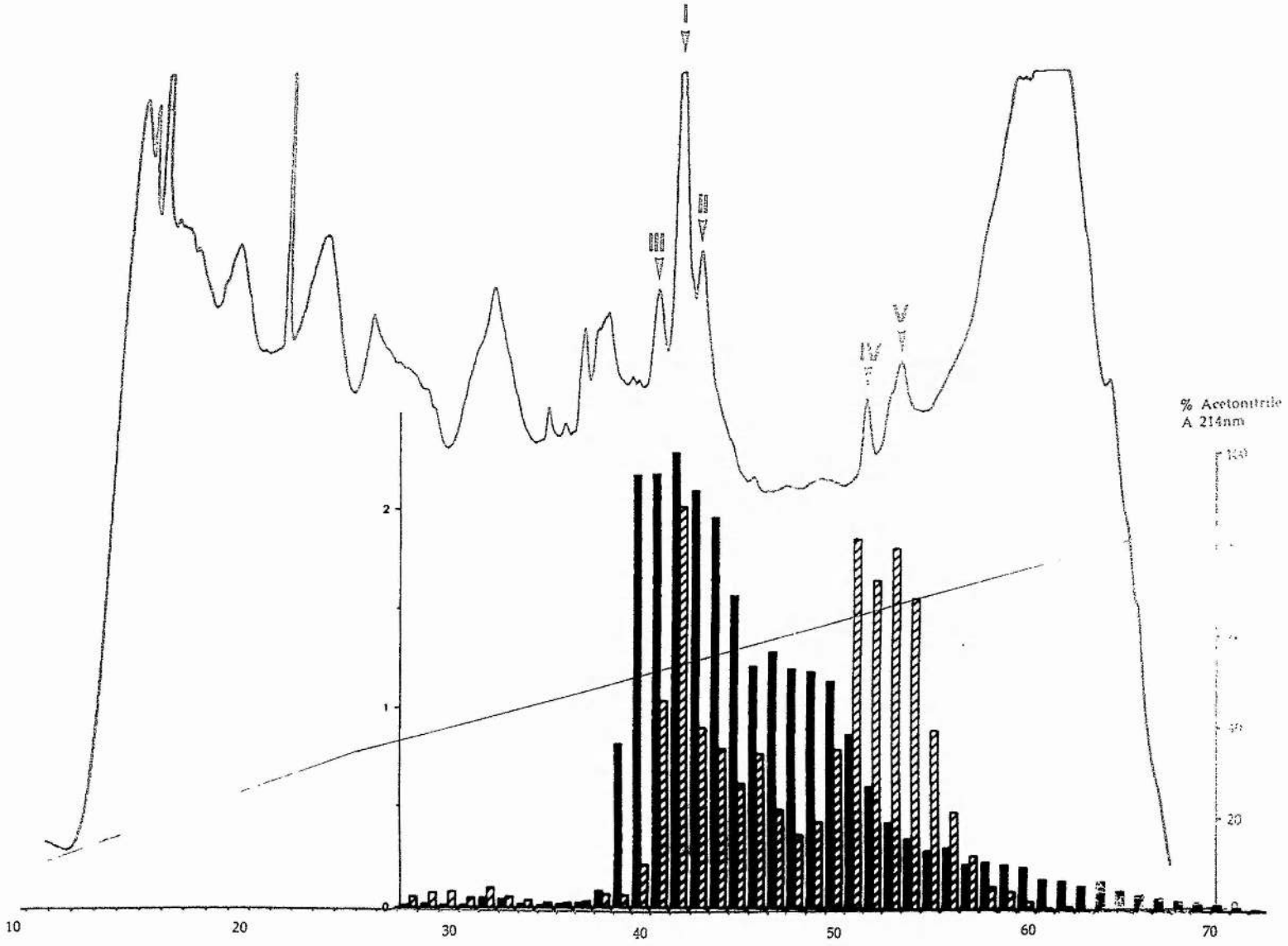
SDS-PAGE of individual fractions obtained from reverse-phase HPLC, followed by Coomassie Brilliant Blue R and then silver staining techniques, was carried out in order to assess the degree of purity of the separated peptides and to estimate their molecular weight according to the migration of markers (figure 4.4). The visualisation of the immunopositive fractions from the propanol gradient (figure 4.2), shows a clear band of approximately 8kDa which corresponds to the fractions with the highest immunoactivity i.e. HPLC fractions 35 and 36 (*cf* figure 4.2). Fractions either side of the maximal immunopositive region show only a faint band. Additionally, HPLC fraction 52, a fraction showing slight immunoactivity, shows a band of approximately 16.5kDa.

Figure 4.3.

Chromatogram obtained by gradient separation reverse phase HPLC of extract of 300 sinus glands on a Capital Nucleosil C-18 column (5µm particle size, 250mm x 46mm internal diameter). Mobile phases were 0.1% TFA (solvent A) and 0.1%TFA in acetonitrile (Solvent B) with a gradient (indicated by a solid line) from 15% B to 85% B and a flow rate of 1.0 $ml.min^{-1}$. U.V. absorbance was monitored at 214nm and fractions were collected at 1 minute intervals.

Figure 4.3.1.

ELISA was performed using the polyclonal rabbit anti-*Orconectes* CHH antisera, represented as solid bars and polyclonal guinea pig anti-*Homarus* GIH antisera, represented as hatched bars. Details of the ELISA protocol and antisera used are described in the text. ELISA was carried out on collected fractions and resulting optical density recorded at 490nm is shown as an superimposed bar graph represented in the text as figure 4.3.1. Maximal absorbance (immunopositive reaction) for the anti-*Orconectes* CHH antisera is associated with a zone including fraction 40–44, while the maximal immunopositive reaction for the anti-*Homarus* GIH antisera is associated with fractions 51–55.


% Acetonitrile
A 214nm
2
1
0
20
10
20
30
40
50
60
70
FRACTION NUMBER

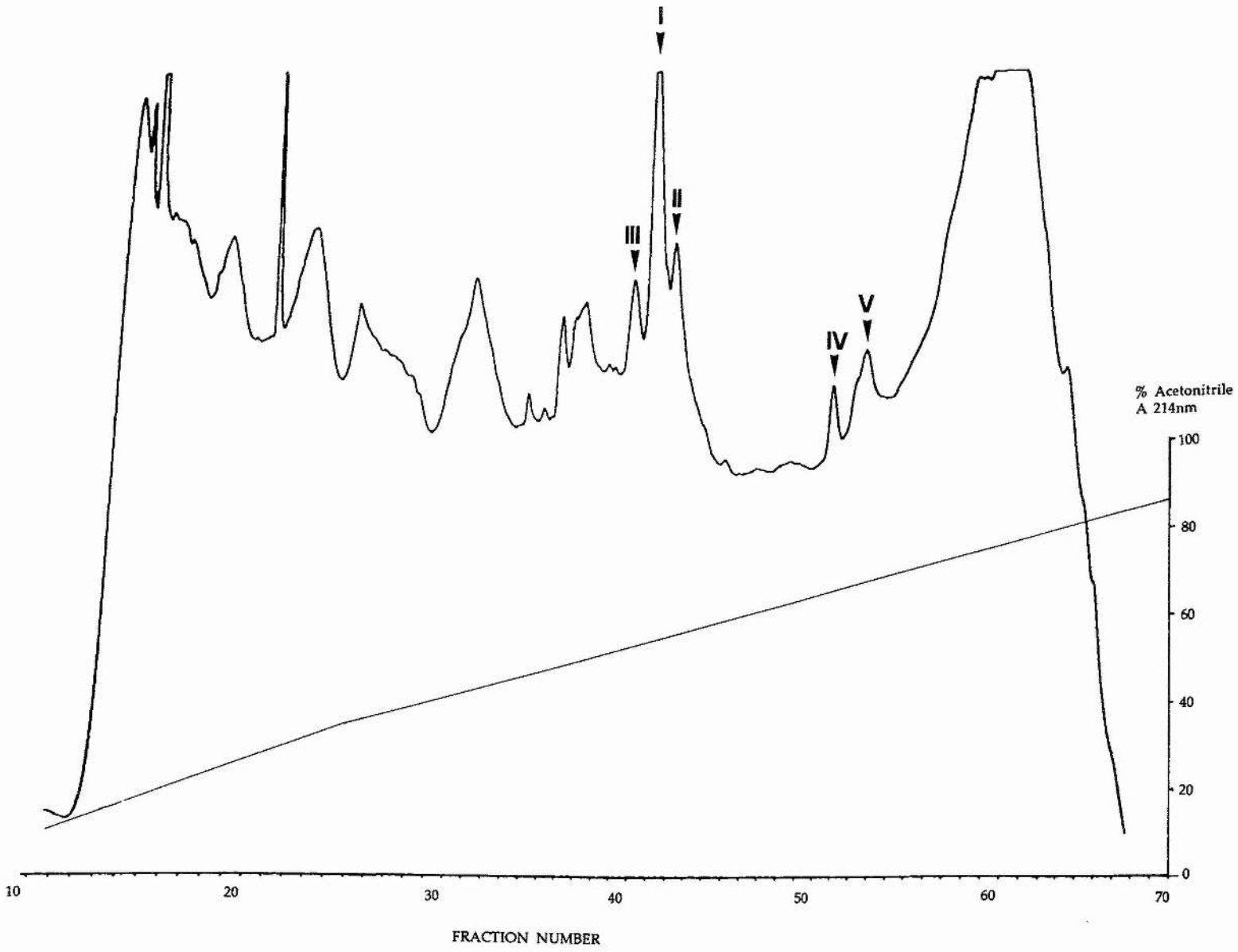

I
II
III
IV
V
% Acetonitrile
A 214nm
100
80
60
40
20
0
10
20
30
40
50
60
70
FRACTION NUMBER

Figure 4.4.

SDS-PAGE gel of immunopositive fractions obtained from HPLC and ELISA (see figure 4.2) stained with the silver staining method described in chapter 2, section 2.15. Fractions 35 & 36 showed a band at around 8kDa and fraction 52 showed a major band at c.16.5kDa. Molecular weight markers (myoglobin polypeptide backbone 1-153, 16950Da; myoglobin I+II 1-131, 14440 Da; myoglobin I+III 56-153, 10600Da; myoglobin I 56-155, 8160 and glucagon 3480 Da Sigma product No G 4250) were run at either side of the gel.

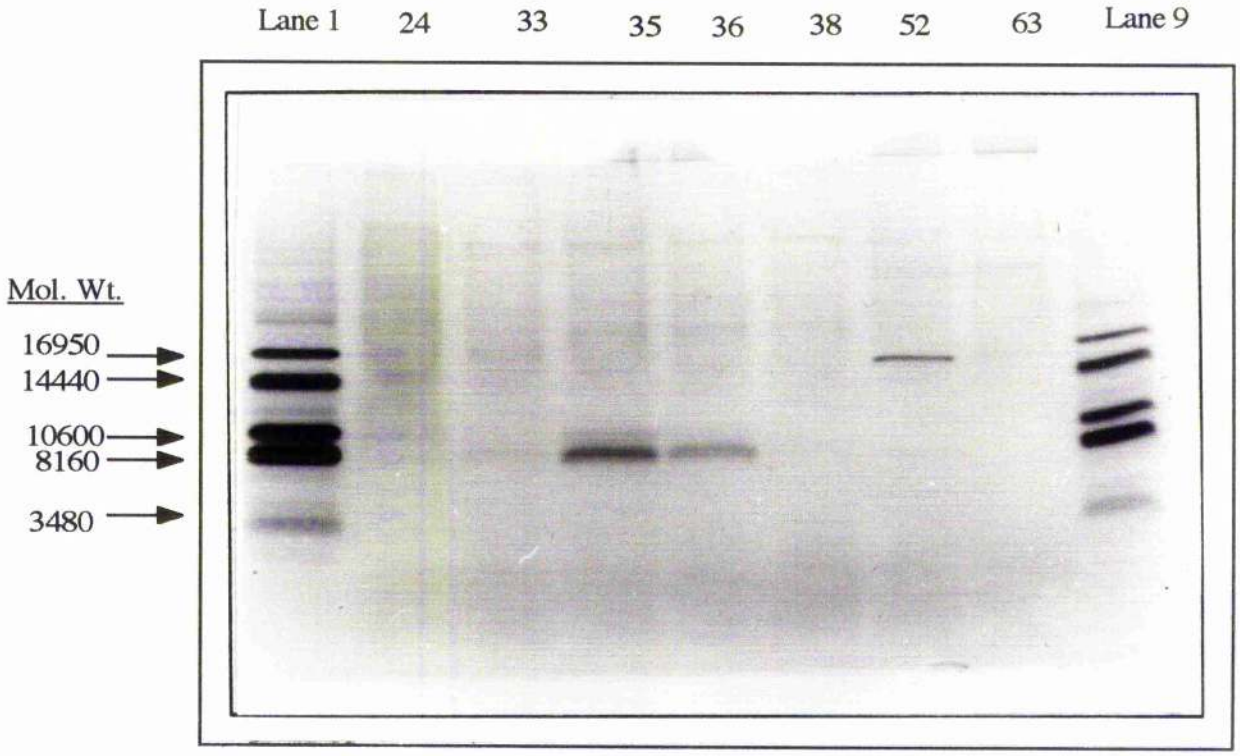
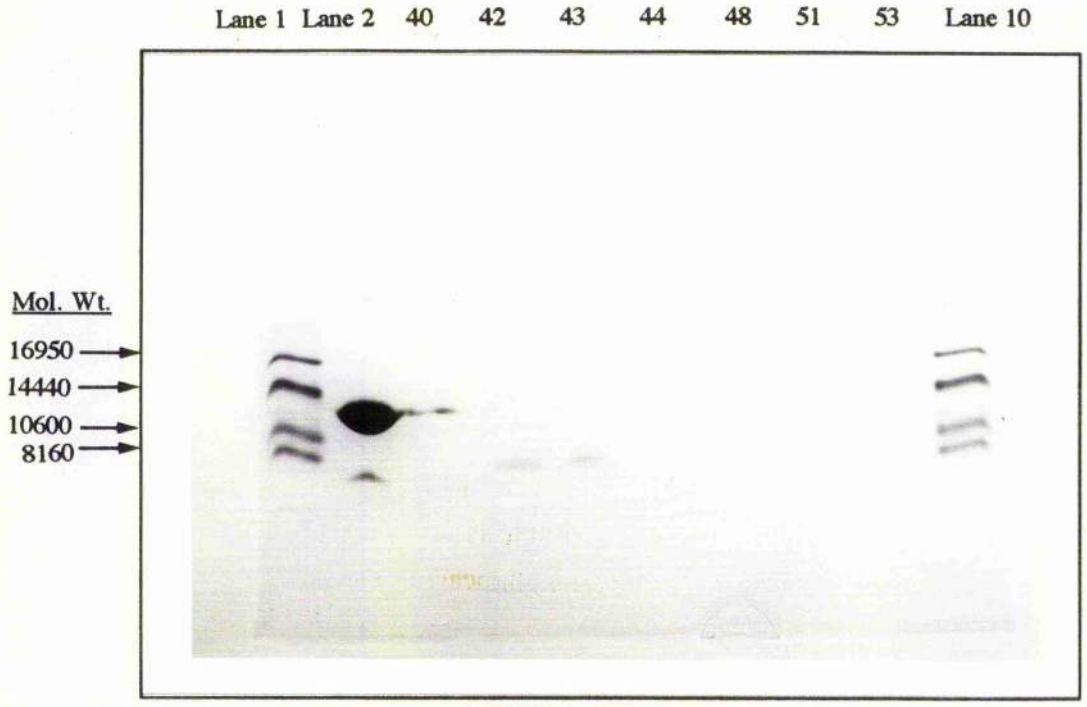

Lane 1
24
33
35
36
38
52
63
Lane 9
Mol. Wt.
16950
14440
10600
8160
3480

Figure 4.5.

SDS-PAGE gel of immunopositive fractions obtained from HPLC and ELISA (see figure 4.3 and figure 4.3.1) stained with the Coomassie Brilliant Blue R method described in chapter 2, section 2.15. Fractions 42 & 43 show a band at around 8kDa, with a loading of approximately 8 sinus gland equivalents. Molecular weight markers (myoglobin polypeptide backbone 1-153, 16950Da; myoglobin I+II 1-131, 14440 Da; myoglobin I+III 56-153, 10600Da; myoglobin I 56-155, 8160 and glucagon 3480 Da Sigma product No G 4250) were run on the far left of the gel. Rabbit parvalbumen was run in lane two as a size marker (main band = 12126Da).

Figure 4.6.

SDS-PAGE gel of immunopositive fractions obtained from HPLC and ELISA (see figure 4.3 and figure 4.3.1). This is the same gel described as figure 4.5, however, staining with the silver stain method described in chapter 2, section 2.15. Fractions 42 & 43 show a band at around 8kDa, however, the band in fraction 42 appears to be slightly larger than that seen in fraction 43. Fractions 51 and 53 show a poorly stained band of approximately 8kDa. The loading is approximately 8 sinus gland equivalents.


Lane 1
Lane 2
40
42
43
44
48
51
53
Lane 10
Mol. Wt.
16950
14440
10600
8160

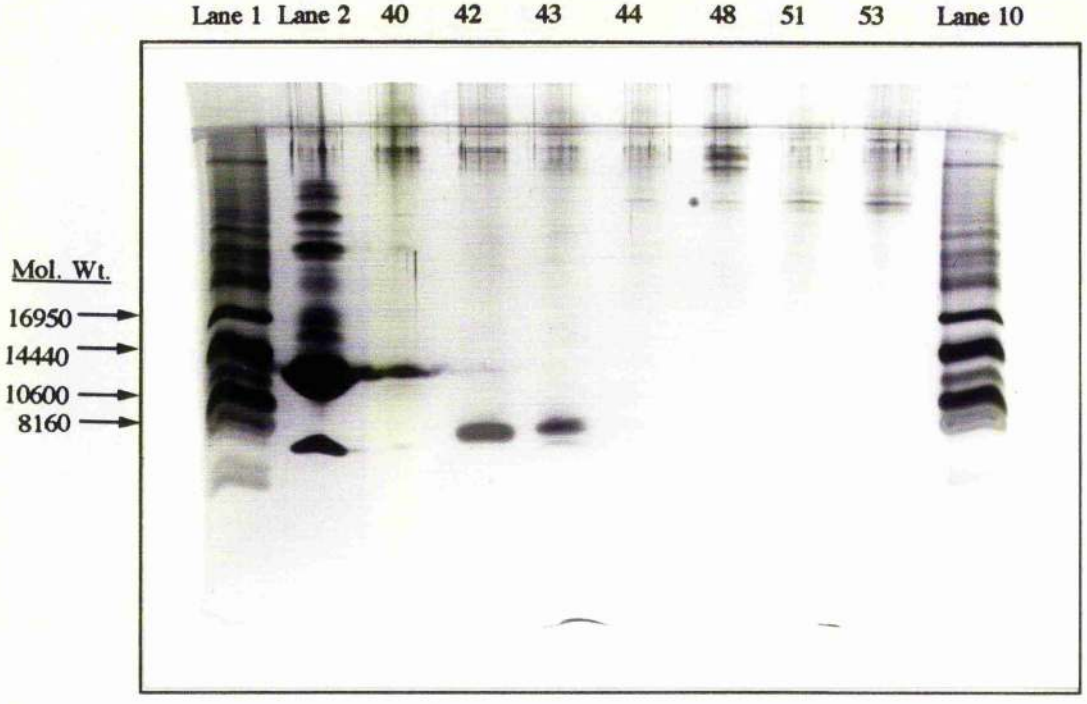
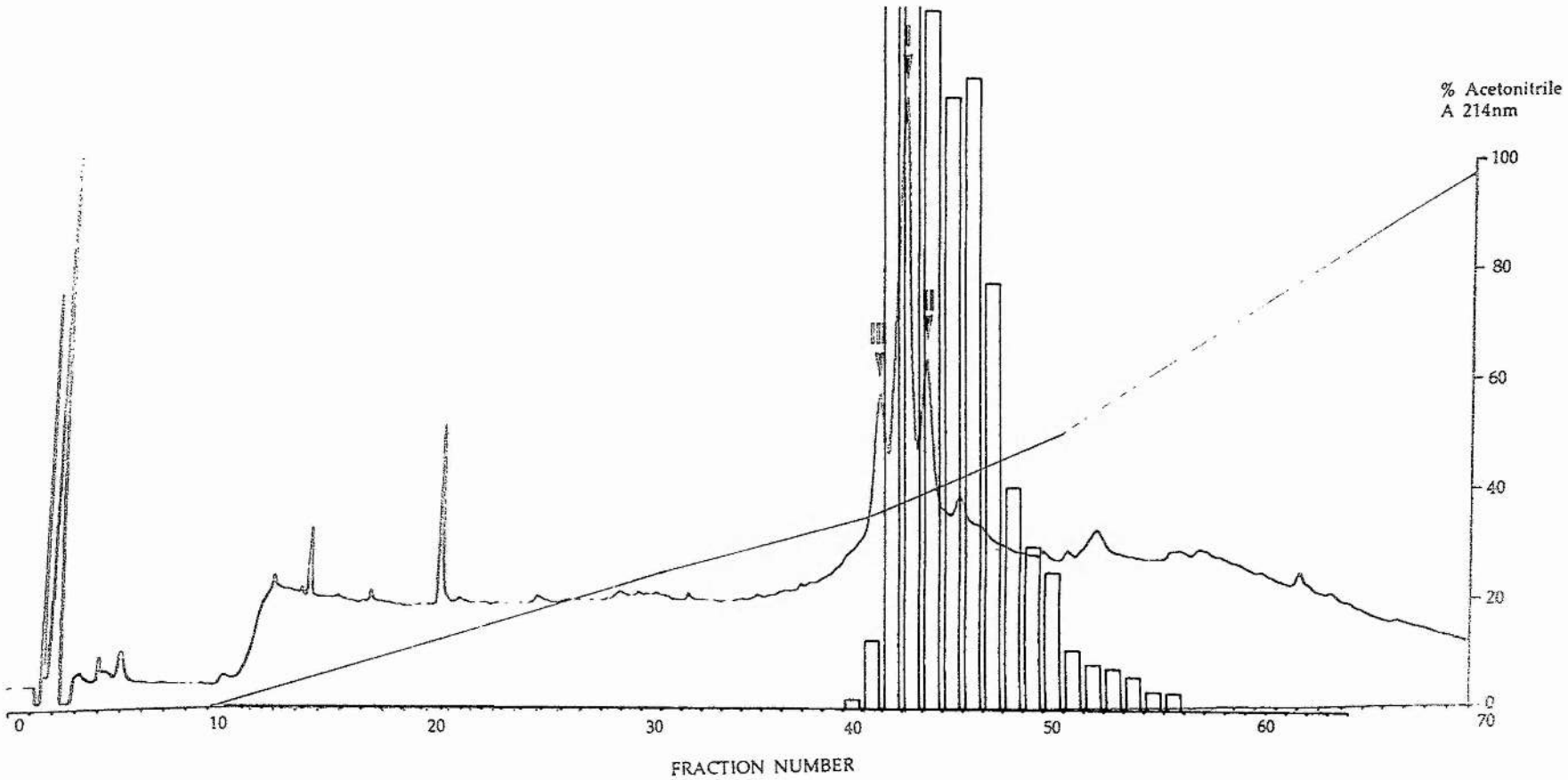

Lane 1
Lane 2
40
42
43
44
48
51
53
Lane 10
Mol. Wt.
16950
14440
10600
8160

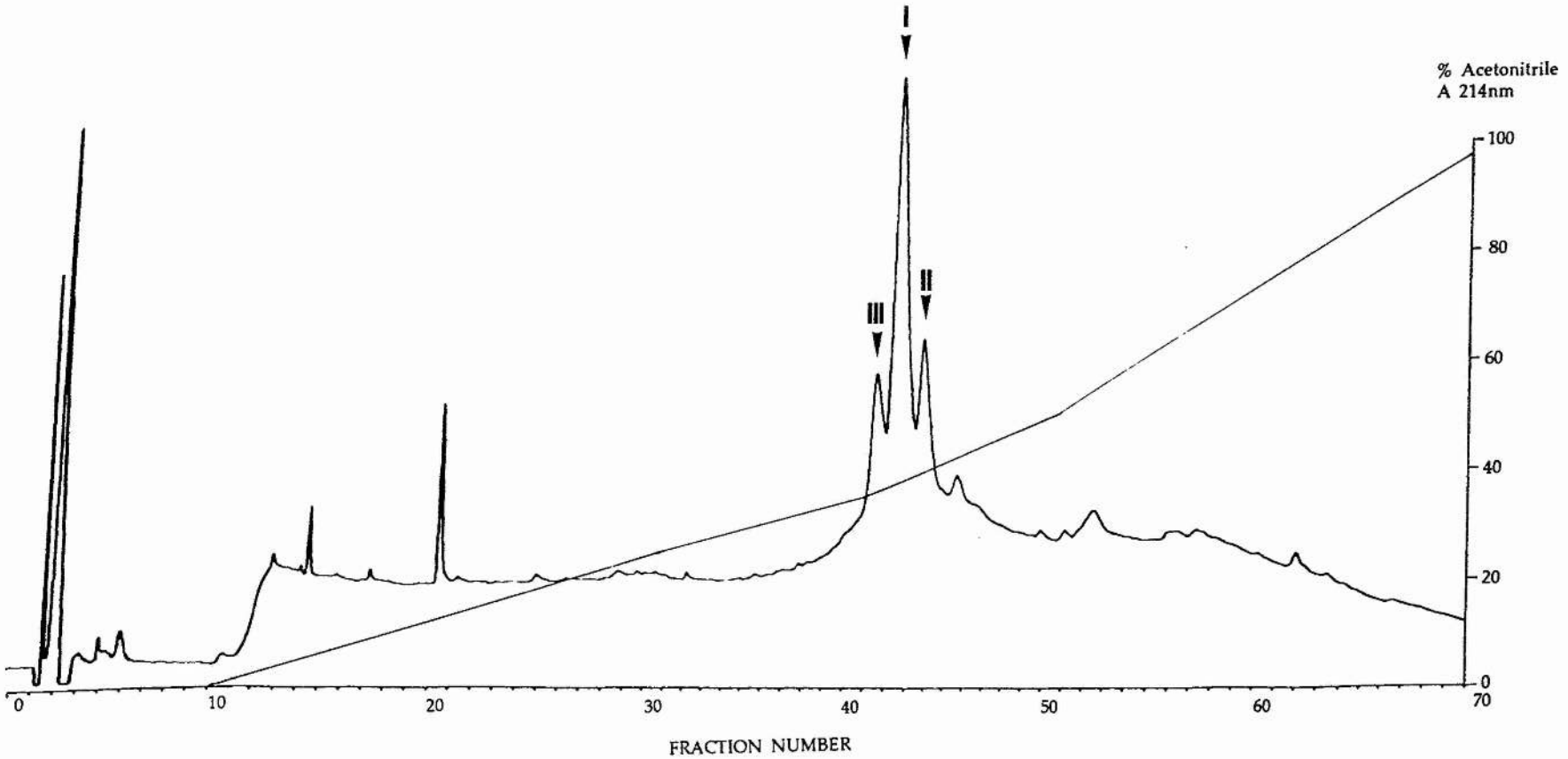
Figure 4.7.

Chromatogram obtained by gradient separation reverse phase HPLC of lyophilised fractions 39–45 obtained by RP HPLC described in figure 4.3. The lyophilised fractions were reconstituted in 175µl and injected into the HPLC system with a Capital Nucleosil C-18 column (5µm particle size, 250mm x 46mm internal diameter). Mobile phases were 0.1% TFA (solvent A) and 0.1%TFA in acetonitrile (Solvent B) with a gradient (indicated by a solid line) from 0% B to 95% B and a flow rate of 1.0 $ml.min^{-1}$. U.V. absorbance was monitored at 214nm and fractions were collected at 1 minute intervals.

Figure 4.7.1.

ELISA using the polyclonal rabbit anti-*Orconectes* CHH antisera, was performed on collected fractions collected from RP HPLC described in figure 4.7 and the resulting optical density recorded at 490nm is shown as an superimposed bar graph. Details of the ELISA protocol and antisera used are described in the text.


% Acetonitrile
A 214nm
100
80
60
40
20
0
0
10
20
30
40
50
60
70
FRACTION NUMBER


I
II
III
% Acetonitrile
A 214nm
100
80
60
40
20
0
0
10
20
30
40
50
60
70
FRACTION NUMBER

Although clear bands are observed, fainter banding is also manifest within each fraction suggesting that the purity is not high and that active fractions should be rechromatographed by a further HPLC step. Immunoactive fractions, including 24, 33, 38, and 63, were run on the same gel, though no bands were apparent following silver staining.

The visualisation of the immunopositive fractions from the acetonitrile gradient (figure 4.3), show a clear band, after staining with Coomassie Brilliant Blue R (figure 4.5) (Neuhoff *et al*, 1988), of approximately 8kDa which corresponds to the fractions with the highest immunoactivity i.e. HPLC fractions 42 and 43 (*cf* figure 4.3.1). The loading on the gel was approximately 8 sinus gland equivalents. It was estimated from the staining of fractions 42 and 43, that the peptide content for a single sinus gland was in the region of 35–125ng.

By using the silver staining method of Bloom *et al* (1987), it became apparent that fractions 42 and 43 (corresponding to peaks I and II) had slightly different molecular weights, although both were approximately 8kDa (figure 4.6). In addition, fainter banding that occurred with this staining procedure demonstrated that a single step purification on the HPLC was not sufficient to obtain a pure sample, although a single HPLC peak was obtained. On closer inspection, very faint banding was noticed from GIH immunoactive fractions 51and 53 corresponding to approximately 8kDa.

4.3.4 Hyperglycaemic bioactivity.

Immunopositive fractions of both HPLC chromatograms were tested for hyperglycaemic activity. Initially, 0.5 sinus gland equivalents of the fractions were used for the bioassay, however, they proved to be individually less potent

than the crude sinus gland extracts that were used as a positive control. The bioassay carried out using fractions collected from the propanol fractionation of sinus gland extracts (figure 4.2), were tested for hyperglycaemic activity, with numbers 35 and 36 showing the maximal hyperglycaemic activity. The other fractions tested, 24, 33, 38, 52 and 63, all showed a non-significant increase in haemolymph glucose and have not been displayed graphically. Figure 4.8 shows the results of the bioassay on fraction 36 and demonstrates that a maximal level of hyperglycaemia occurs after the injection of approximately 4 sinus gland equivalents.

The data obtained from the ELISA of the HPLC fractions (figures 4.3 and 4.3.1) demonstrate highly comparable results between the immunopositivity of the fractions and the hyperglycaemic response. Fraction 32 shows no significant immunoactivity to either CHH or GIH antisera, however, fractions 41, 42, 43, and 48 each show an immunoactive response, with fraction 42 displaying maximal immunoactivity to both CHH and GIH. Fraction 48 is immunopositive to CHH, although the HPLC chromatogram shows no major peaks. Fractions 51 and 53 (peak IV and peak V) are immunoactive mainly to GIH, although the anti-CHH response is at a much reduced level. Bioassay of these two fractions failed to increase circulating glucose significantly. These data are summarised in figure 4.9.

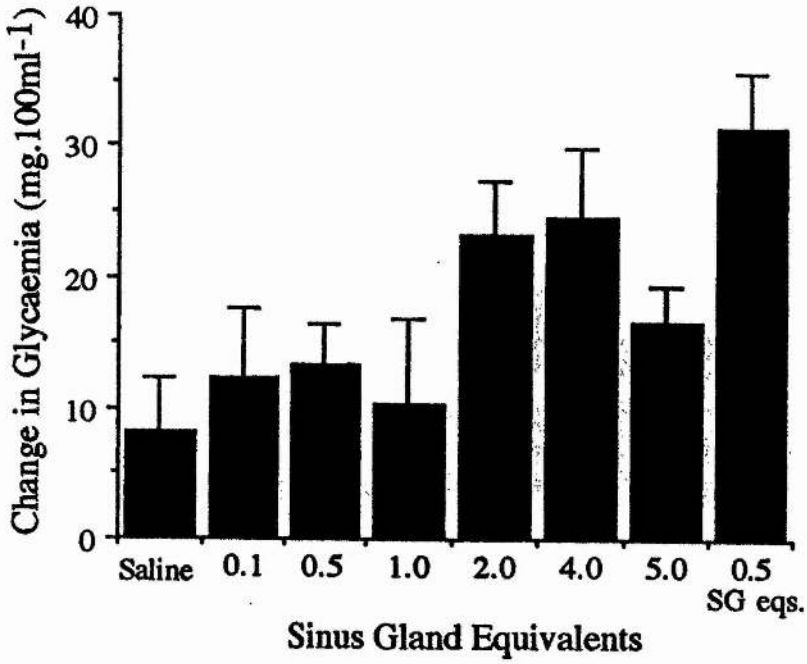
These results clearly demonstrate fraction 42 (peak I) is the major hyperglycaemic fraction, although the hyperglycaemic activity of fractions 41 (peak III), 43 (peak II) and 48 is significantly lower than this main fraction. Fractions 51 and 53 (peak IV and V) which are highly immunoactive to the GIH antisera, show a non-significant increase of haemolymph glucose, although, fraction 42 (peak I), which demonstrates a high affinity to both antisera, does contain hyperglycaemic activity.

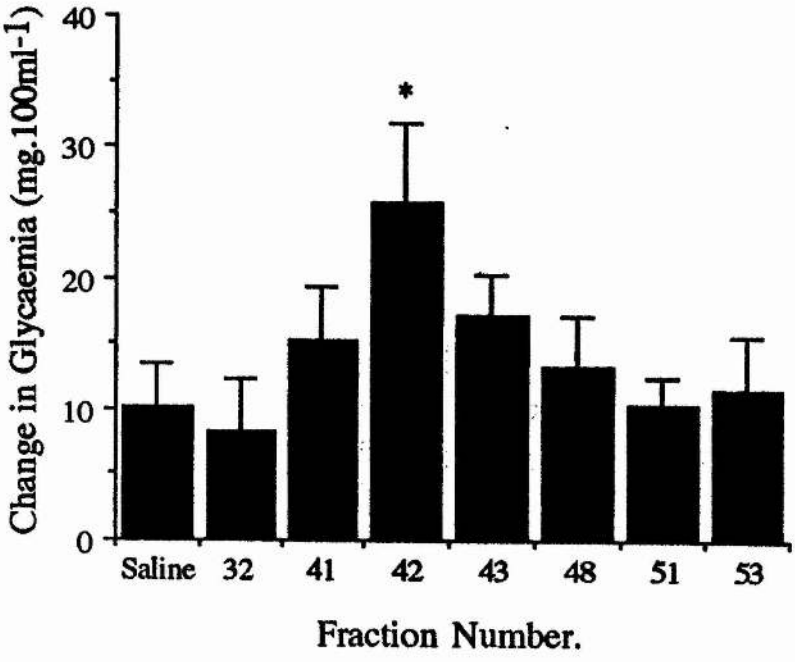
Figure 4.8.

Hyperglycaemia in *Nephrops norvegicus* obtained using anti-*Orconectes* CHH immunoactive fraction 36 collected from the propanol fractionation of sinus gland fractions by RP HPLC (see figure 4.2) at varying concentrations of sinus gland equivalents. A negative control of saline injections is shown on the left of the figure and positive control of 0.5 sinus gland equivalents are shown on the right. All values show change in glycaemia which is expressed as $mg.100ml^{-1}$.

Figure 4.9.

Changes in haemolymph glycaemia in *Nephrops norvegicus* following injection of RP HPLC purified sinus gland extracts (see figure 4.2). Prior to injection, fractions were lyophilised and reconstituted in saline. Four sinus gland equivalents of each fraction shown, were each injected into five animals. All values show change in glycaemia in $mg.100ml^{-1}$. Significant differences compared to the initial glucose concentrations were determined by means of the Students *t*-test and are indicated by asterisks: * = $p<0.05$.


Change in Glycaemia (mg.100ml-1)
40
30
20
10
0
Saline
0.1
0.5
1.0
2.0
4.0
5.0
0.5
SG eqs.
Sinus Gland Equivalents


Change in Glycaemia (mg.100ml-1)
40
30
20
10
0
*
Saline
32
41
42
43
48
51
53
Fraction Number.

The protein determination of this single fraction, which displayed both CHH immunopositivity and hyperglycaemic activity, was calculated to be 66.25ng.sinus gland^{-1} (Smith *et al*, 1985). This is consistent to the estimation of peptide content determined from the Coomassie Brilliant Blue R stained SDS-PAGE gel of the HPLC fractions, 42 and 43. According to the terminology proposed by Raina and Gäde (1988), this peptide, fraction 42 (peak I) has been designated putative *Nephrops norvegicus* crustacean hyperglycaemic hormone (*Nen*-CHH).

4.3.5 Antigen/antibody calibration for *Nen*-CHH.

The dilution of fraction 42 (peak I), the putative *Nen* - CHH, is shown in figure 4.10. These data demonstrate that one sinus gland equivalent of the HPLC fraction is the most effective concentration of antigen, while higher concentrations of antigen caused an inhibitory effect on the ELISA. This optimal antigen concentration does not however negate the earlier ELISA reactions which used HPLC fractions of approximately 0.5 sinus gland extracts equivalents. The previous results are valid as the method was utilised to identify active fractions obtained by the fractionation of sinus gland extracts. Furthermore, the reaction for this experiment was stopped approximately 5 minutes earlier than the usual ELISA of HPLC fractions, in order that the absorbance at 490nm could be measured. From these results, it is possible to speculate that the anti-*Orconectes* CHH antisera produces an immunoactive response with as little as 33ng of *Nen*-CHH. Results obtained later in this thesis suggest that the antisera may be able to detect lower levels of the peptide (see Chapter 7)

Figure 4.10.

ELISA using the polyclonal rabbit anti-*Orconectes* CHH antisera, was performed on a sinus gland equivalent dilution gradient of fraction 42 collected from RP HPLC described in figure 4.3 and the resulting optical density recorded at 490nm. Details of the ELISA protocol and antisera used are described in the text.

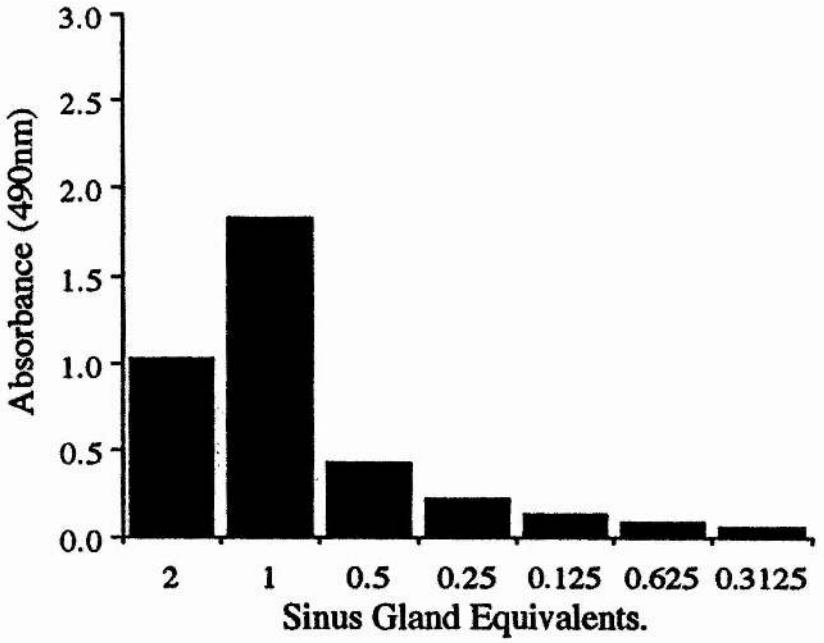
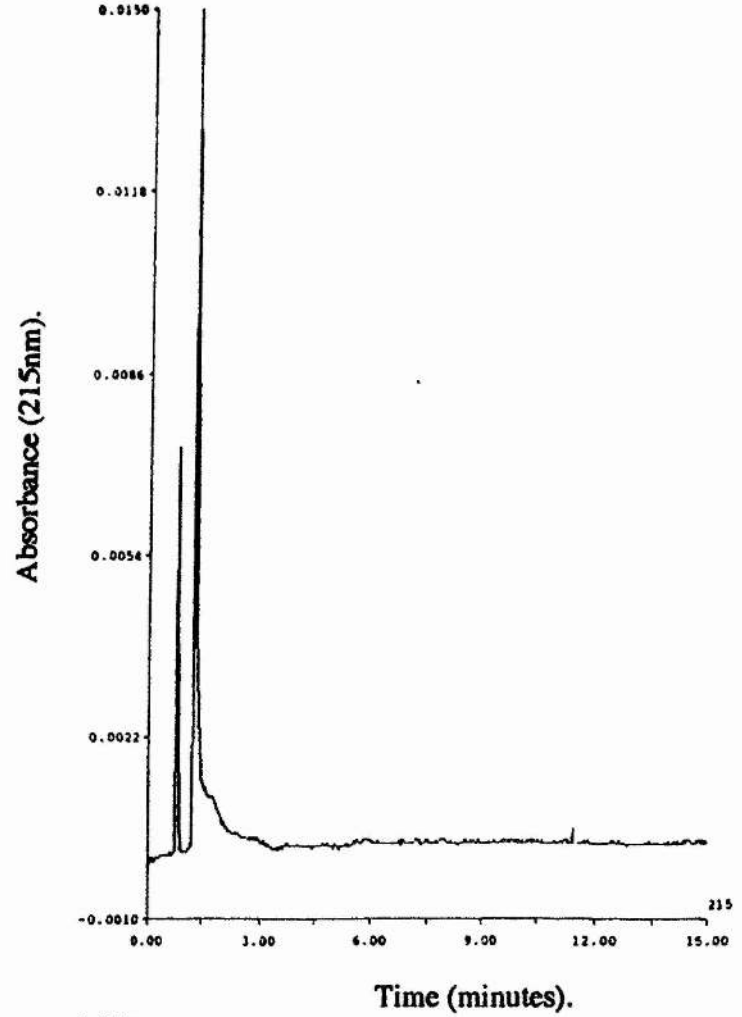

3.0
2.5
2.0
1.5
1.0
0.5
0.0
Absorbance (490nm)
2
1
0.5
0.25
0.125
0.625
0.3125
Sinus Gland Equivalents.

Figure 4.11.1

Capillary electrophoresis (CE) chromatogram of RP HPLC fraction 42 (see figure 4.4). A major peak is observed after approximately 1.5 minutes with a minor contaminating peak occurring slightly earlier. The sample is run at an absorbance of 200nm. Details of the CE protocol are described in chapter 2, section 2.16.

Figure 4.11.2

Capillary electrophoresis (CE) chromatogram of RP HPLC fraction 42 (see figure 4.4) at an absorbance of 215nm.


0.0150
0.0118
0.0086
0.0054
0.0022
-0.0010
215
0.00
1.00
6.00
9.00
12.00
15.00
Absorbance (215nm).
Time (minutes).

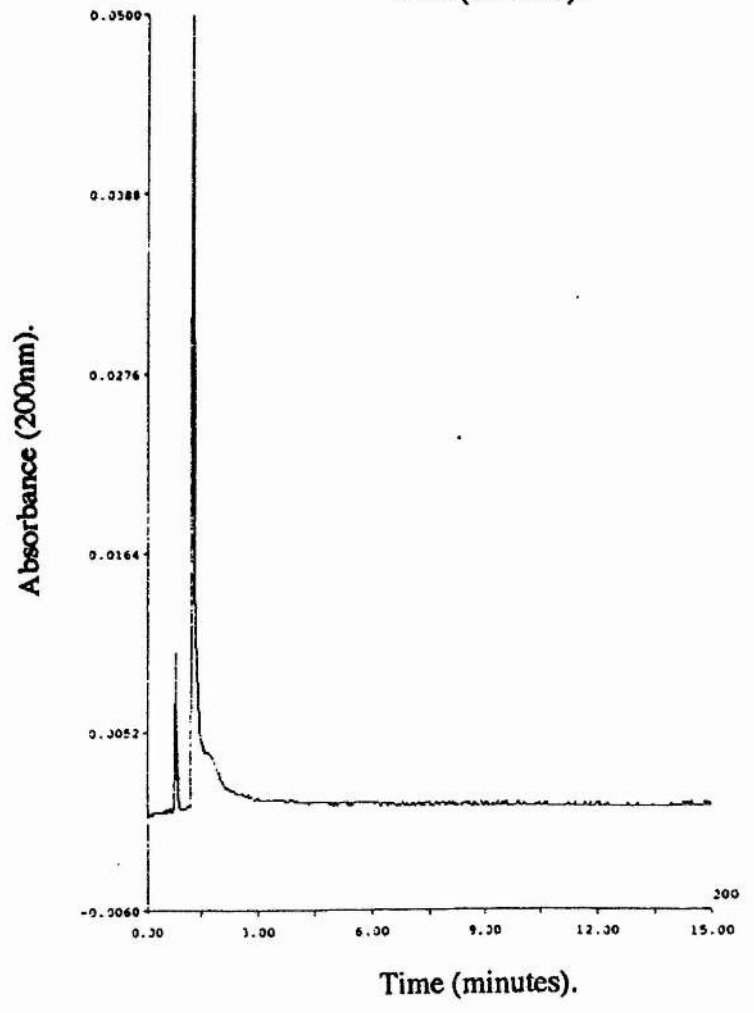
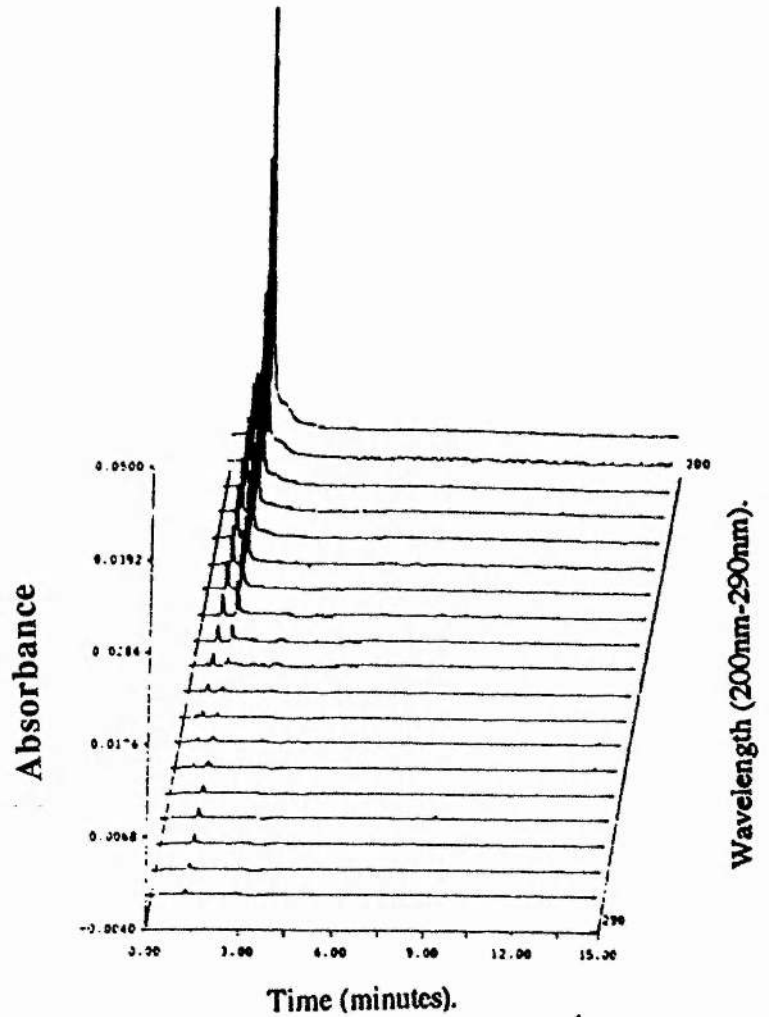

0.0500
0.0388
0.0276
0.0164
0.0052
-0.0060
200
0.00
1.00
6.00
9.00
12.00
15.00
Absorbance (200nm).
Time (minutes).

Figure 4.11.3

Capillary electrophoresis (CE) chromatogram of RP HPLC fraction 42 (see figure 4.4) with a scanning absorbance from 200nm to 290nm. The event time scale is the same as in figures 4.11.1 and 4.11.2 i.e. 0–15 minutes.

Figure 4.11.4

Capillary electrophoresis (CE) chromatogram of RP HPLC fraction 42 (see figure 4.4) with a scanning absorbance from 200nm to 290nm. The event time is set from 0–1.5 minutes in order to obtain a better separation of the peaks. The major peak can be seen to occur after 1.3 minutes, while the minor peak occurs at 0.8 minutes.


Absorbance
Wavelength (200nm-290nm).
Time (minutes).

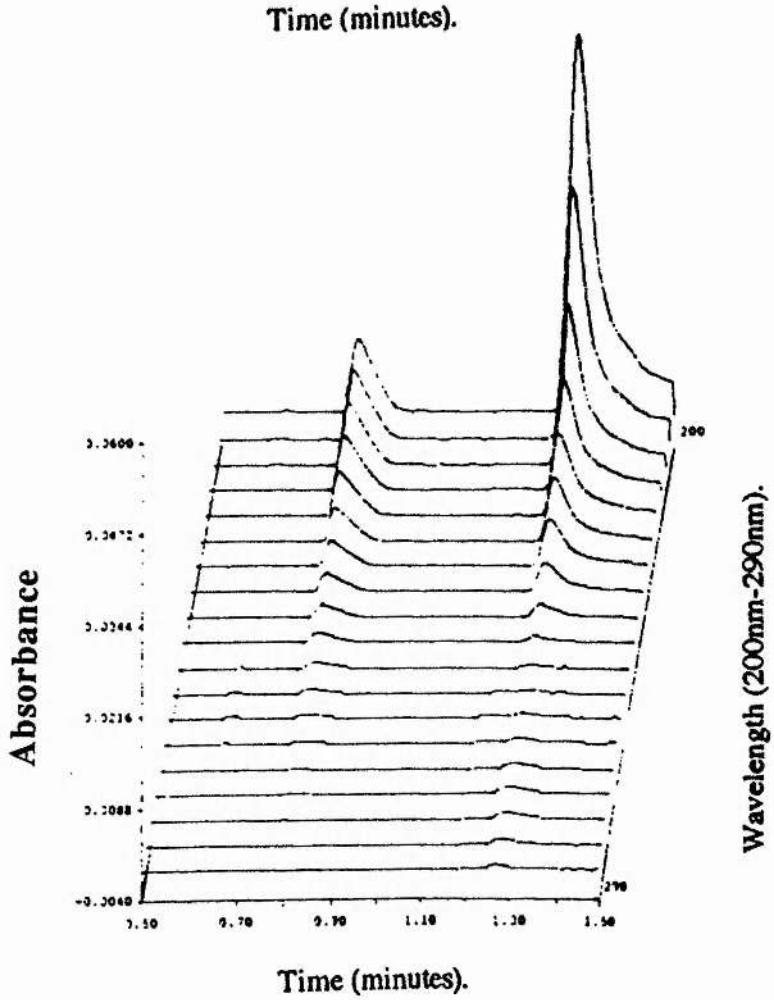
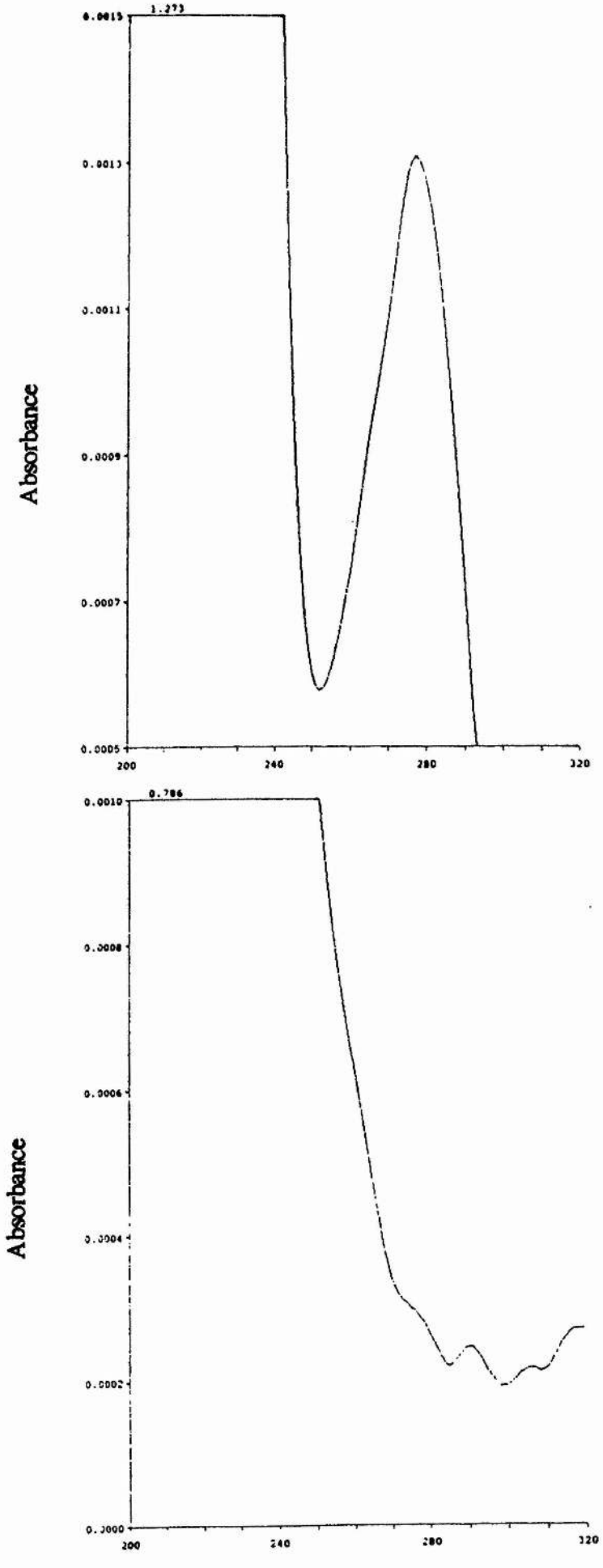

Absorbance
Wavelength (200nm-290nm).
Time (minutes).

Figure 4.11.5.

Capillary electrophoresis (CE) chromatogram of major peak shown in figure 4.11.4 obtained from RP HPLC fraction 42 (see figure 4.4) with a scanning absorbance at 280nm.

Figure 4.11.6.

Capillary electrophoresis (CE) chromatogram of minor peak shown in figure 4.11.4 obtained from RP HPLC fraction 42 (see figure 4.4) with a scanning absorbance at 280nm.


1.273
0.0015
0.0013
0.0011
0.0009
0.0007
0.0005
Absorbance
200
240
280
320
0.786
0.0010
0.0008
0.0006
0.0004
0.0002
0.0000
Absorbance
200
240
280
320
Wavelength (280nm).

4.3.6 Capillary electrophoresis.

Following the second HPLC purification step of fractions 39-45, described in section 4.3.2 above, the three peaks were clearly separated (figure 4.7). Bioassay confirmed the results of figure 4.9, that fraction 42 (peak I) was the most significantly hyperglycaemic fraction. The purity of the fraction was assessed using capillary electrophoresis.

The use of capillary electrophoresis (CE) demonstrates that fraction 42 contained a single peptide peak which absorbed both at 200nm (figure 4.11.1) and 215nm (figure 4.11.2). A smaller contaminating peak is also detected within this fraction, which elutes just before the major peak. When the fraction is scanned from 200–290nm, it can be clearly observed that the major peak absorbs through the spectrum (figure 4.11.3 and 4.11.4), but importantly, the peptide absorbs at 280nm (figure 4.11.5) confirming the presence of a protein. The contaminating peak however, is not detected at 280nm which suggests that it is non-protein in nature (figure 4.11.6).

4.3.7 Amino acid sequencing.

Initial attempts to obtain N-terminal amino acid sequences for putative *Nen*-CHH were unsuccessful., as no amino acids were released using automated Edman degradation (Edman and Begg, 1967). The two step HPLC purification described above was repeated and the fractions corresponding to *Nen*-CHH (fraction 42, peak I), and the fractions 51 and 53 (peak IV and peak V) were collected and lyophilised prior to Edman degradation. The purity of the sample was not assessed by CE. The protein content of putative *Nen*-CHH was estimated to be approximately 12-16μg, equivalent to 250 sinus glands.

Prior to sequencing the *Nen*-CHH sample was applied to a Vydac C18 column which resulted in a chromatogram showing seven peaks. A small quantity of the major component (fraction 6) was injected into the sequencer and ran for 12 cycles. A probable sequence for the N-terminus of the protein is shown below:

---

1 5 10 12
Ser-Leu-Val-Phe-Lys-Ala-Lys-Arg-Phe-Arg-Phe-Val....

It was not possible to detect any peptide in the fractions 51 and 53. This may be due to the peptides high hydrophobicity and that they became irreversibly bound to the plastic tubes in which they were lyophilised.

The Edman degradation was carried out by Dr. J. Michael Conlon at Creighton University, Nebraska, U.S.A., to whom I am indebted.

4.4 Discussion.

The use of Sep Pak, as previously discussed was used only to determine the concentration of solvent which would elute the hyperglycaemic peptide the most effectively. Due to the reports that peptides can be irreversibly bound to the support medium (Keller and Kegel, 1984), this method was not used as a initial purification step. Following the employment of a Sep Pak cartridge (Waters), in order to ascertain at what percentage solvent CHH active material elutes from a C18 reverse phase support, it was found that the majority of hyperglycaemic activity was associated with a 25% acetonitrile wash. An

acetonitrile gradient was therefore created with a gradual solvent change from 16% to 30%, thus allowing the greatest recovery of peptide, while at the same time allowing any differences in hydrophobicity of the peptides to be utilised to separate peaks of interest. Following the HPLC purification of the sinus gland extract, it was determined that the CHH immunoactive fractions were apparent between 22 and 35% acetonitrile.

After fractionation of the *Nephrops norvegicus* sinus gland extracts by RP HPLC utilising a propanol gradient, only a single peak gave a strong immunoactive reaction to the anti-*Orconectes* CHH antiserum, although areas of weak immunoactivity were also observed. It is possible that these areas may be due to cross-reactivity of the antiserum with GIH or MIH. There does not appear to be the same degree of polymorphism for CHH that has been described in *H. americanus* and *O. limosus* (Tensen *et al*, 1989; Soyez *et al*, 1990; Tensen, 1991), and although the immunoactive peak identified in this study is clearly asymmetric, the extent of polymorphism described in the above animals is not apparent in *N. norvrgicus*. The Mexican crayfish, *Procambarus bouvieri*, has been shown to have two isoforms, CHH-I (formerly named CHH-B) which is the major isoform, and CHH-II, the minor isoform, which has a later elution (Huberman *et al*, 1992; 1993). The HPLC elution profile for sinus gland neuropeptides from *P. bouvieri*, shows four highly hydrophobic peptides designated GIH, MIH, CHH I, and CHH II in order of elution (Aguilar *et al*, 1992). The GIH immunoactive fractions obtained from *N. norvegicus* appear to be more hydrophobic than the CHH active fractions. It would be interesting to bioassay these later peaks, peak IV and peak V obtained from the actetonitrile gradient for GIH activity, and likewise to assess the MIH immunoactivity and bioactivity of peak III.

In figure 4.2 of this study, the shape of the first peak suggests there may in fact be two peptides with similar immunoactivity, although SDS-PAGE

indicates identical molecular masses for both adjacent fractions. The use of an acetonitrile gradient produces a similar result, though the bioactivity can, following a second HPLC purification step, be associated mainly with a single peak, peak I (figure 4.7). Although the immunoactivity occurs after the active peak, the hyperglycaemic activity of the adjacent fractions is low. It is possible that the adjacent peak in figure 4.7 is a further CHH peptide, although its activity is very low. As separation of these peaks could not be easily obtained due to their similar hydrophobicities, it is considered that this activity is possibly due to the "bleed" of the putative *Nen*-CHH as the solvent concentration rises. However, the possibility that peak I is *Nen*-CHH I and that peak II is *Nen*-CHH II cannot be ignored. Interestingly, SDS-PAGE and the silver staining technique, indicates that although these peptides have a molecular mass of approximately 8kDa, they do appear to have slightly different masses. This could be confirmed by FAB/MS.

In other studies the use of a two step HPLC protocol has been developed separating peptides by differences in their hydophobicity and their ionic charge (Tensen *et al*, 1989; Soyez *et al*, 1990). The use of ionic and hydrophobic separation was attempted with *Nephrops* CHH, however, the recovery of peptide was extremely low, and was therefore not considered to be of use in this study. The reasons for this are unclear. The two step HPLC isolation used in this study separated peptides only by their hydrophobicity, with the peaks separated and isolated using a shallower and longer gradient of acetonitrile in the region of interest.

It should be noted that there appears to be differences between the antigen binding in this study and that described by Tensen (1991) between the *Orconectes* CHH antigen and the anti *Astacus* CHH antiserum. Further differences between the antigen binding to the anti-*Orconectes* CHH antisera and the bioassay have been demonstrated. The hyperglycaemic bioassay employed

in this study suggests that 0.5 sinus gland equivalents is the optimum concentration for the reaction (figure 4.1), though the bioassay concentration required to obtain a significant increase of haemolymph glucose from HPLC fractions, was 2–4 equivalents (figure 4.8 and 4.9). The use of the ELISA on the dilution gradient of the single active fraction (figure 4.10) suggests that the optimum concentration is 1 sinus gland equivalent. As discussed in Chapter 1, antisera may have a specific binding site particular for a single peptide, however, all or part of this binding site could be recognised by a region of a peptide, resulting in an immunopositive reaction. These differences of antigen/antisera binding in this study, compared to that of others, could only be confirmed by raising a *Nephrops* specific antiserum.

Work by Meusy and Soyez (1991) shows differences between repeated ELISA experiments. It was considered that inaccuracy could develop in stored samples that had been frozen and thawed, this action making the reaction much more sensitive. This may explain some of then variation between ELISA concentration gradient and bioassay gradient developed in this study. The injection of higher than expected levels of HPLC purified fractions, in order to obtain a maximal hyperglycaemic response in recipient animals, was clearly seen in figure 4.8. This effect has been previously documented in the crayfish, *Orconectes limosus* (Kegel *et al*, 1991; Tensen, 1991), and the lobster, *Homarus americanus* (Tensen *et al*, 1989; Tensen, 1991).

The results obtained from the acetonitrile fractionation of sinus gland extracts (figure 4.3), demonstrate the separation of immunoactive peaks for both the anti CHH and the anti GIH antisera. The anti-*Orconectes* CHH can be seen to react maximally to the three closely related peaks, between fractions 39 and 45, while the anti-*Homarus* GIH reacts both with this region and a later region, fractions 51–54 (figure 4.3.1). These results are similar to those obtained by Meusy and Soyez (1991). It was demonstrated that a number of different

peptides, representing the CHH groups I and II and the GIH groups, shared at least one identical epitope and that CHH and GIH are not species specific. In this study the use of anti-*Orconectes* CHH and anti-*Homarus* GIH antisera on *Nephrops norvegicus* antigen, clearly demonstrates that the epitope for the immune reaction of both CHH and GIH is not species specific. Structural analogies may be reasonably expected to exist, at least within the Astacidea, and data from a number of authors confirm this phenomenon (Van Deijnen, 1986, Tensen *et al*, 1989). Finally, the occurrence of an anti-GIH response in both the CHH and the GIH active areas, while the anti-CHH reaction is restricted purely to the CHH region, indicates that HPLC purified peptides can be split into two groups; a CHH group and a GIH group. Differing results were obtained by Meusy and Soyez (1991), where the anti-GIH antiserum only produced an immunopositive reaction with the GIH peptide, while the anti-CHH antiserum reacted with all the tested HPLC purified peptides. It appears therefore, that the epitope that is recognised by the anti-CHH antiserum is different to that of the anti-GIH antiserum. This immunoactive response of the anti-GIH antibody with both the CHH and the GIH epitope will be discussed further in Chapter 7.

As discussed earlier in this section, the analysis of the molecular weights using SDS-PAGE of the immunoactive fractions, suggested a single band obtained by HPLC of around 8kDa for both solvent methods used. This suggests that a peptide isolated in this study appears to be of similar size to CHH peptides in other species. It should be noted, however, that Chang *et al* (1987) and Soyez *et al* (1990) both indicated that SDS-PAGE led to an underestimation of molecular weight determination when compared to values obtained using FAB/MS (Fast Atom Bombardment Mass Spectrometry). *Homarus americanus* has a CHH II of 8578Da and a CHH VII of 8656Da (Tensen *et al*, 1991b; c), while Soyez *et al* (1990), deduced molecular masses for four basic CHH isoforms, also from *Homarus*, of 8577Da and 8633Da. *Penaeus vannamei* has a

hyperglycaemic peptide of 8895Da (Soyez *et al*, 1992), *Procambarus bouvieri* of 8300–8400 (Huberman *et al*, 1992) and the terrestrial isopod *Armadillidium vulgare* of 8729Da (Martin *et al*, 1992). The identification of an 8kDa peptide in this study (figure 4.4, 4.5 and 4.6) is therefore consistent with the molecular weight of CHH from other species. The HPLC fraction 52, displayed only slight immunoactivity, contains a staining band of approximately 16.5kDa (figure 4.4). It is possible that this may be the inactive precursor hormone, pre-pro CHH, of *Nephrops norvegicus*. Clearly at this stage, this is speculative. However, the preprohormone of *Cardisoma carnifexis* was identified as a predominant 14kDa prohormone (Stuenkel, 1986) and in *Carcinus maenas*, as 13.5kDa (Weidemann *et al*, 1989) which have similar molecular masses to the HPLC fraction 52 obtained in this study.

Other peptides with apparent CHH activity have also been demonstrated to have GIH (gonad-inhibiting hormone) activity (Tensen *et al.*,1989), while a CHH active moult-inhibiting peptide (Chang *et al.*, 1987; 1990) has a similar sequence to those described as *Homarus* CHH I/II (Tensen, 1991; Tensen *et al*, 1991b), *Carcinus maenus* CHH (Kegel *et al*, 1989) and *Orconectes* CHH (Kegel *et al*, 1991). The GIH immunoactive fractions identified in this study did not show significant hyperglycaemic study, a result which appears to differ from those above. However, faint bands of approximately 8kDa could be obtained by SDS-PAGE (figure 4.6), although the peptide content of these fractions is very low. Further investigation is required into the role of GIH in *Nephrops* before any valid conclusions can be made.

Silver staining of the propanol fractions indicates that the fractions are not totally pure following a single HPLC step. A second acetonitrile step was thus utilised and the purity was confirmed using CE. To the knowledge of this author, this is the first time that CE has been used for the purification of eyestalk neuropeptides. It is a quick, simple and non-destructive method of determining,

not only the purity of a sample, but also the molecular mass and PI of a peptide. Furthermore, it can be used for the optimisation of PCR.

It is proposed that the hyperglycaemic peptide, described in this chapter, which was isolated from the sinus glands of *Nephrops norvegicus*, be termed putative *Nephrops norvegicus* crustacean hyperglycaemic hormone, *Nen*-CHH, to be confirmed following successful sequence analysis.

The sequence obtained in this study and shown in section 4.3.7 has virtually no homology to any CHH isoforms sequenced to date, furthermore, there is little homology to the published sequences of GIH or MIH from any other species. The HPLC trace which was made prior to sequencing indicates that the fraction containing peak I obtained by the two step fractionation of *Nephrops norvegicus* sinus glands was not pure and that it contained seven possible peptides. The purity of the fraction was not determined by capillary electrophoresis prior to Edman degradation. As the sequence obtained could not be aligned against any point of the other neuropeptide sequences so far deduced, it is not likely that the seven peaks observed are the denatured products of *Nen*-CHH. Unless the sequence of CHH from *Nephrops norvegicus* is totally distinct from that of any other CHH isoforms published thus far, which is unlikely, then the peak that has been N-terminally sequenced, is a contaminant. The demonstration in this chapter that both the anti-*Orconectes* and anti-*Homarus* antisera are capable of immunopositive activity with *N. norvegicus* sinus gland extracts and HPLC fractions, indicates that there is probably a high degree of homology of CHH sequences between these species. Furthermore, the ability of degenerated oligonucleotide primers specific to regions of *Homarus* CHH to successfully amplify cDNA from *N. norvegicus* total eyestalk RNA (see Chapter 6) indicates that there must be a high sequence homology between the CHH of both species.

Chapter 5.

Endocrine responses to photoperiodicity and physiological stress in *Nephrops norvegicus*.

5.1 Introduction.

Many behavioural and physiological processes are influenced by circadian rhythms, and within the Crustacea, these rhythms have been linked to neurosecretory release, with the secretion of neurohormones from the XOSG complex being controlled by environmental influences. Figure 1.3 in Chapter 1 indicates the major external influence on the eyestalk and subsequent endocrine release, two of which are photoperiod and physiological stress. Rhythms of neurosecretion in crustaceans are observed both in intact and eyestalk ablated animals and it is clear, therefore, that the entire neurosecretory system and not only the eyestalk are responsible for the maintenance of endogenous cycles (Aréchiga *et al,* 1985; Keller and Orth, 1990). An attempt has been made in this study, to identify any circadian rhythmicity of circulating glucose in *Nephrops norvegicus* and to determine the role of CHH in the associated hyperglycaemia in relation to photoperiodic influence and physiological stress (severe hypoxia).

Crustacean hyperglycaemic hormone synthesis and release has been shown in certain species to be subject to circadian rhythmicity (Hamann, 1974; Kallen *et al,* 1990). CHH neuropeptides are produced by the XOSG complex and are involved in a number of physiological processes (Van Herp and Kallen, 1991). Although the role of CHH in the regulation of carbohydrate metabolism although first demonstrated by Abramowitz in 1944, it was not until 30 years later that Hamann established that the sinus gland was responsible for the maintenence of an endogenous rhythm of circulating glucose. From these experiments it was concluded that the role of CHH was not only for the maintenance of glucose levels, but possibly as a provider of available glucose supplies during periods of physiological demand (Keller *et al,*1985; Keller and Sedlmeier, 1988).

The energetics of CHH producing cells in the eyestalk have revealed that the cells themselves demonstrate a circadian rhythmicity, with CHH released at the onset of darkness and again, though of a smaller magnitude, at the onset of the light period (crepuscular secretion). Both periods are preceded by a peak of synthetic activity (Gorgels–Kallen and Voorter, 1985; Kallen *et al*, 1988). Dopamine, serotonin and Met-enkephalin are thought to be involved in the regulation of CHH synthesis and release, mediated by synaptic input of CHH axon ramifications in the medulla terminalis (Gorgels–Kallen, 1985; Van Herp and Kallen, 1991). Observations that serotonin and dopamine can only evoke hyperglycaemia in intact and not destalked animals and that Met-enkephalin decreases circulating glucose and abolishes diurnal glucose rhythms, seems to support this synaptic input hypothesis (Kallen *et al*, 1988). Tensen (1991) demonstrated an increase of synthetic activity, with a peak of CHH mRNA occurring 2 h prior to the release of CHH at the start of the dark period. In addition, the demonstration of CHH mRNA expression in the cerebral, thoracic and abdominal ganglia, indicate that the eyestalk may not be the only site of CHH release. Furthermore, this may explain why destalked animals had detectable circulating CHH in heat stressed *Orconectes* (Keller and Orth, 1990).

The role of stress in crustaceans and its effects on the endocrine system, have received little attention. Moreover, it seems that stress hyperglycaemia has been an encumbrance for the researchers attempting to isolate, characterise and sequence CHH. The most recent work by Keller and Orth (1990), Santos and Keller (1992) and Smullen and Bentley (1992), have demonstrated the complexities of this area of investigation. Work presented in this chapter, is aimed at providing a basis for further physiological investigations of stress on the endocrine system of *Nephrops norvegicus*. The role of photoperiod in an animal whose environment extends from shallow water to extreme depths and

the effects of anoxia on a species that regularly experience such conditions is of great interest.

5.2 Materials and Methods.

5.2.1 Identification of a circadian rhythm of glucose in *Nephrops norvegicus*.

Specimens of *Nephrops norvegicus* were obtained by local fisherman and maintained in the laboratory as described in Chapter 2. The animals were starved and maintained on a 12L:12D regime (0800-2000h) for four days prior to experimentation. Two days before the start of the experiment, six healthy males of approximately equal size and weight (30–40mm carapace length, 20–30g) were separated from each other by placing them into individual containers. Two 50μl haemolymph samples were obtained from the animals every 4 h, starting at 1200hrs for a period of 28 h. The haemolymph samples were added to equal volumes of anticoagulant, mixed gently and then stored at -20°C until required. Bioassay was carried out on one sample, as described in Chapter 2, in order to determine the circulating glucose levels, while circulating CHH levels were determined by using the second sample in a direct ELISA using anti-*Orconectes* CHH antisera. During the dark period, haemolymph samples were collected from animals under a red light in order that there should be no naturally occurring light during this period which may alter any endogenous rhythm of circulating CHH and glucose.

5.2.2 Physiological stress imposed on *Nephrops norvegicus* by severe hypoxia.

External environmental factors have been shown to alter the levels of circulating CHH (Keller and Orth, 1990). The effect of severe hypoxia (7 torr) was investigated in *Nephrops norvegicus*, by the immersion of two groups of animals, one intact and one with their eyestalks ablated, into deoxygenated sea water. A third group were immersed in normoxic conditions. Each group contained six males of approximately the same size and weight (30–40mm carapace length, 20–30g). 50μl haemolymph samples were taken at 5, 15 and 20 minutes intervals and at 1, 2, 5, 8, and 12 h after immersion, mixed with equal volumes of anticoagulant and haemolymph glucose was determined by the bioassay method discussed in Chapter 2. Deoxygenation of the sea water in which the animals were maintained, was obtained by the constant bubbling of nitrogen through the water, the surface of which was covered in a thin plastic sheet in order to prevent the surface diffusion of oxygen. The oxygen content of the water was determined by the use of the Winkler method. Prior to and throughout the experiment, the O_2 levels were continuously monitored in order that the same level of hypoxia was maintained. The normoxic conditions were obtained by the a constant flow of sea water into a tank which was continuously aerated. The temperature of both the hypoxic and the normoxic conditions was recorded throughout the experiment and found to remain constantly between 8-10°C.

5.3 Results

5.3.1 Changes in glucose levels during a day/night cycle.

Initially it was thought that the results obtained from this experiment showed that there was no endogenous circadian rhythm of glucose in *Nephrops norvegicus* and that each animal had what appeared to be random levels of circulating glucose. However, upon more detailed examination, it was noticed that three of the six experimental animals showed a degree of rhythmicity and three animals apparently displayed no such cycle. This result is shown in figure 5.1 where the glucose levels of individual animals are displayed as a line extending over the time period for the experiment, with plot marks denoting the sample points. The solid lines indicate the three animals that showed a glycaemic rhythm, while the three animals represented as a dotted line, apparently did not show any circadian rhythmicity.

Clearly the experimental animals can be divided into rhythmic and arhythmic individuals, the former of these (solid lines), showing an increase of glucose 4 h prior to the onset of darkness and with the largest value of glucose occurring at 2000hrs. By 0000hrs the glucose levels had decreased markedly, but by the onset of the light period at 0800hrs, the glucose levels had increased significantly. It should be noticed however, that the levels of glucose following this second elevatory period, did not drop to values observed at the start of the experiment. For example, at 1200hrs on day one, the glucose level were significantly lower than 1200hrs on the second day. Alternatively, the circulating glucose levels at 1600hrs on both days were not significantly different.

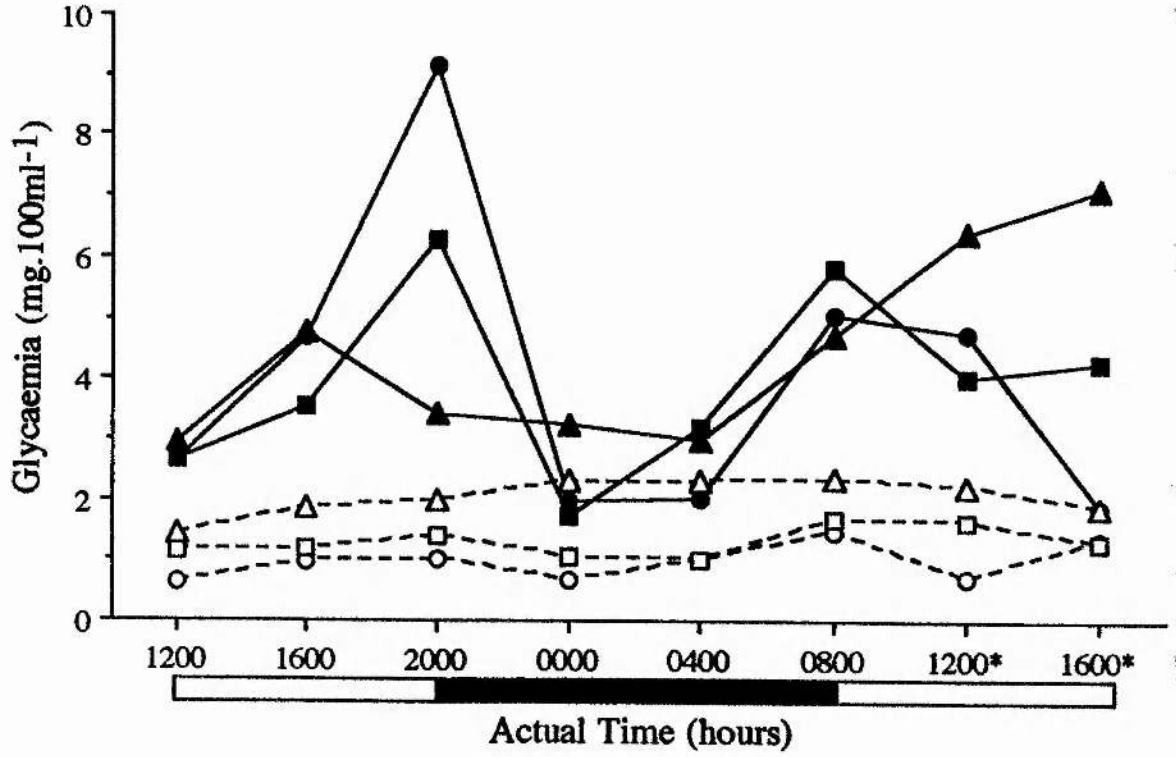
The animals that were separated into the arhythmic group (dotted lines) showed a low level of glycaemia, which did not significantly differ throughout

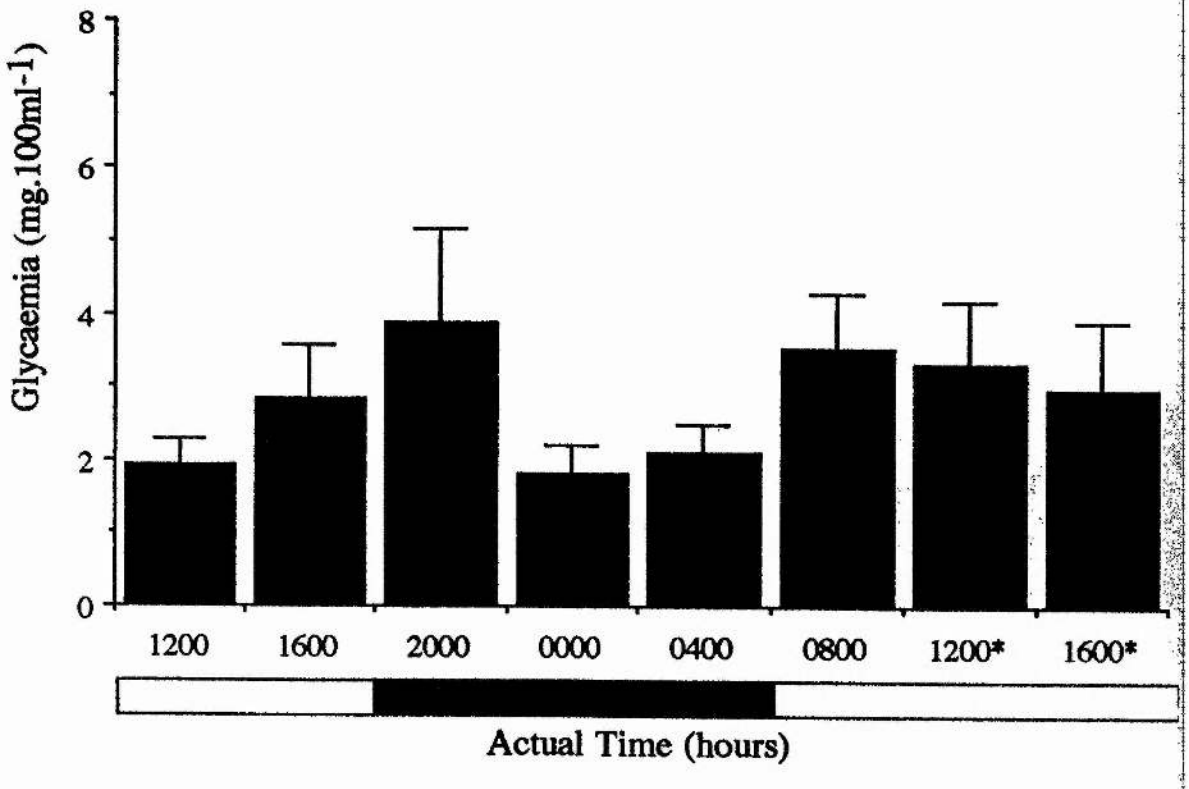
Figure 5.1.

Haemolymph glucose (mg.100ml^{-1}) levels during a 28-h period, determined for individual *Nephrops norvegicus* (n=6) kept under constant 12 h light/12 h dark; lights on at 0800. Solid lines indicate animals displaying rhythmicity, dashed lines indicate animals displaying no rhythmicity (see text for details). Box below x axis depicts light and dark periods.

Figure 5.2.

Haemolymph glucose levels of *Nephrops norvegicus*, mean ± sem, n=6 (mg.100ml^{-1}) during a 28-h period, kept under constant 12 h light/12 h dark; lights on at 0800. Students *t*-test shows no significant difference between any group at $p = <0.05$. Box below x axis depicts light and dark periods.


Glycaemia (mg.100ml-1)
10
8
6
4
2
0
1200
1600
2000
0000
0400
0800
1200*
1600*
Actual Time (hours)


Glycaemia (mg.100ml-1)
8
6
4
2
0
1200
1600
2000
0000
0400
0800
1200*
1600*
Actual Time (hours)

the experimental period. In addition, the error terms were low and stable, unlike the error terms expressed in the animals displaying glucose rhythmicity. Interestingly, the initial levels of circulating glucose in each of the groups are different, approximately $1mg.100ml^{-1}$ in the arhythmic group and nearly $3mg.100ml^{-1}$ in the group displaying rhythmicity. The reason for this difference is not clear. The data presented in figure 5.2 is the mean value for all the animals (n=6) and demonstrates that the variation is not significantly apparent in the experimental population.

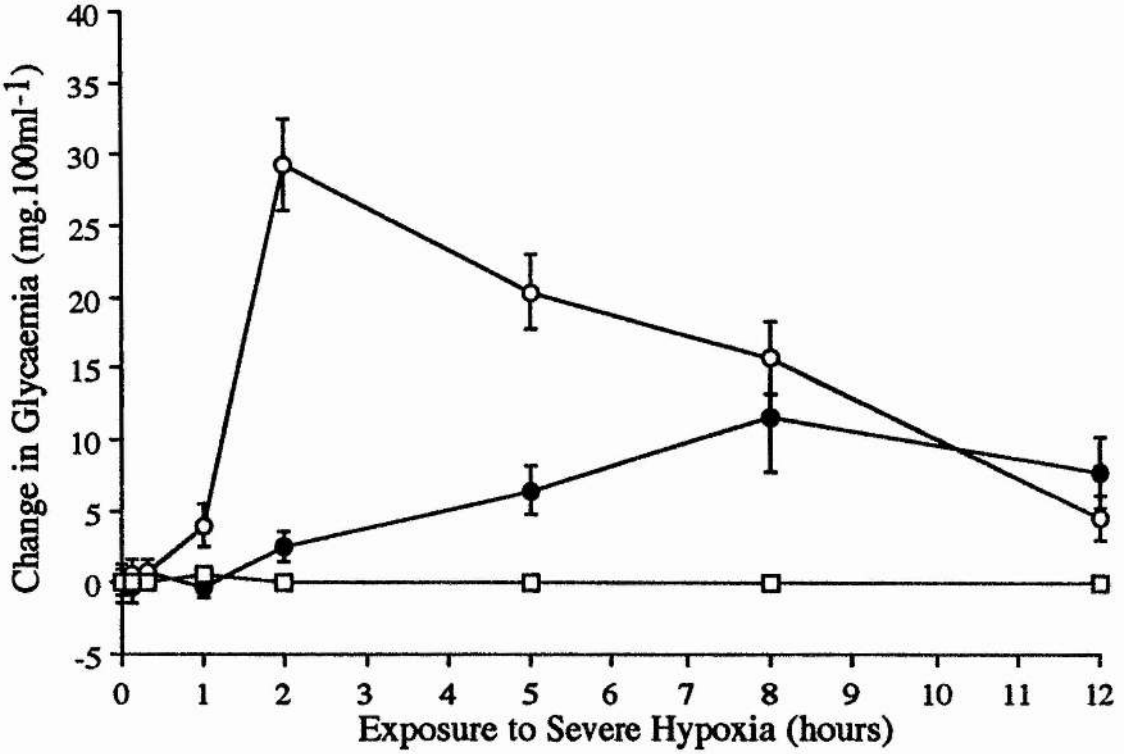
The use of ELISA was not successful in determining the levels of circulating CHH in *Nephrops norvegicus* and the data are not presented. The antibody, however, reacted immunopositively with all samples, quickly over staining each haemolymph sample. It appeared that the use of a single antibody is not sufficient, partly as the levels of CHH in the haemolymph are very low, but in addition, the level of non-specific binding of the antibody is very high, even when the antigen samples were blocked with B.S.A. or a 3-5% powdered milk solution. It is proposed that a second CHH specific antibody is required in order to carry out a antigen capture assay or double antibody sandwich ELISA (DAS–ELISA) as adopted by Gorgels–Kallen and Voorter (1985) for the measurement of circulating CHH in the haemolymph of *Astacus leptodactylus*.

5.3.2 Effects of severe hypoxia on circulating glucose levels in *Nephrops norvegicus*.

Figure 5.3 shows change in glycaemia values at different times after immersion of *N. norvegicus* into a severely hypoxic environment. Animals maintained under hypoxia showed an increase of glycaemia after an hour and this reached a maximum of $30mg.100ml^{-1}$ after two hours. Over the subsequent ten hours the glucose levels were reduced to values measured at the start of the

Figure 5.3.

Haemolymph glucose levels of *Nephrops norvegicus* during 12 h immersion in severely hypoxic sea water (7 torr). Open circles represent intact animals maintained under severe hypoxia, closed circles represent intact animals maintained under normoxic conditions and open squares represent eyestalk ablated animals maintained under severe hypoxia. For each treatment group n=6 mean ± sem. Following immersion, haemolymph samples were taken after 5 minutes, 20 minutes, 40 minutes, 1 h, 2 h, 5 h, 8 h, and 12 h. Glycaemia depicted as change in glycaemia in mg.100ml^{-1}.


Change in Glycaemia (mg.100ml-1)
40
35
30
25
20
15
10
5
0
-5
0
1
2
3
4
5
6
7
8
9
10
11
12
Exposure to Severe Hypoxia (hours)

experiment. Animals that had been eyestalk ablated showed no increase in haemolymph glucose concentration over the whole experiment, while in intact *N. norvegicus* under normoxic conditions, there was only a small increase in glucose levels over the course of the experiment which was maximal after eight hours. It should be noted that all the experimental animals survived the 12h period of deoxygenation, these included the six animals that had their eyestalks ablated.

5.4 Discussion.

The physiological effects of altered environmental conditions and the metabolic involvement of CHH in Crustacea in response to these situations, is a complex area of study. The resulting "stress hyperglycaemia", although documented has rarely been investigated in detail. Results presented in chapter 3 demonstrate that the effect of handling, and the associated increase of circulating glucose in *Nephrops norvegicus* is not significantly great unless animals are injected repeatedly to remove haemolymph samples (figure 3.14). Independent groups of animals could be sampled to avoid this effect (Keller and Orth, 1990), however, if independent groups were to be sampled in this study, highly variable basel glucose levels in *N. norvegicus* would necessitate a high sample size in order to negate any inter-individual variation. The high levels of hyperglycaemia measured in other decapods during handling stress (Riegel, 1960; Telford, 1968) are not apparent in *N. norvegicus* unless they are treated in the manner described above. The diurnality of glucose and CHH has been demonstrated (Hamann, 1974; Kallen, 1988, 1990; Keller and Orth 1990), as have CHH mRNA in the crayfish, *Orconectes limosus* (Tensen, 1991), although other environmental conditions have not received a great deal of attention. Keller and Orth (1990) demonstrated the relationship of CHH with other environmental conditions, including feeding and starvation, diurnality, the effect of temperature

elevation and the response to anaerobism. The effect of anaerobism on crustaceans is well documented (see Chapter 1), however, the association of CHH to this stressor has not been investigated in detail.

Physiological work reported here has shown a difference between *Nephrops norvegicus* and *Orconectes limosus* with regard to their responses to severe hypoxia (Keller and Orth, 1990; Smullen and Bentley, 1992). The hyperglycaemic response to anoxia exhibited by *O.limosus* (Keller and Orth, 1990) showed an increase of circulating glucose over a 12 hour period whereas the results obtained from *N. norvegicus* clearly showed a decrease after two hours to a point that basel levels of circulating glucose were obtained 12 hours later. In both species, basel levels of glucose were maintained for the first hour, although Keller and Orth (1990) have identified a peak of CHH release during this period. It has been suggested that the glucose metabolism during this initial hour is rapid, thus preventing a significant build-up in the haemolymph. Following this idea, *N. norvegicus* appears to utilise the circulating glucose more efficiently as a source of fuel for anaerobic metabolism which has been shown to result in the increased production of L-lactate (Spicer *et al*, 1990; Smullen, unpublished data). Effectively, hyperglycaemia incurred during severe hypoxia may be related to the greater need for an increased supply of substrate for glycolysis Unfortunately, data for CHH levels in *N. norvegicus* are not available at this time, although further information is hoped to be presented at a later time.

The techniques described here were being adopted to elucidate if this contrast between two species within the Astacidea could be linked to differing habitats. *Nephrops norvegicus* clearly experience hypoxia within its burrow on regular occasions and actively circulates water, by increasing the beat frequency of the pleopods and gill balers when the pO_2 of the surrounding water drops below a specific point (Taylor, pers. comm.; Hagerman and Uglow, 1985;

Atkinson and Taylor, 1988). As *N. norvegicus* also resorts to anaerobic metabolism during periods of activity (Spicer and Hill, unpublished observations) this will effectively utilise the increased glucose levels and consequently result in a decrease in circulating glucose. The physiological and behavioural adaptations of *N. norvegicus* to hypoxic conditions has been discussed in detail in the introduction to this thesis (section 1.1.3.3)

In this present study and in that of Keller and Orth (1990) destalked animals showed no increase of haemolymph glucose during exposure to hypoxic conditions. This would suggest that initially the source of the glucose in the intact animals would be by the hydrolysis of glycogen to glucose and the resynthesis of glycogen from free haemolymph oligosaccharides or the hydrolysis of haemolymph oligosaccharides to glucose (Meenakshi and Scheer, 1961; Scheer and Meenakshi, 1961).

Taylor and Spicer (1987) demonstrated the ecological significance of the differing abilities of *Palaemon elegans* and *P. serratus* to survive hypoxic exposure. *P. elegans* are found in rock pools and are thus exposed to a wide range of physiological extremes, of which hypoxia is one (Morris and Taylor, 1985), while *P. serratus* is essentially a sub-littoral species and encounters less extreme environments (Rodriguez and Naylor, 1972). Under anoxic conditions *P. elegans* showed an increase of blood glucose over the first two hours, although by the fourth hour the glucose levels had been significantly reduced (Taylor and Spicer, 1987). *P. serratus* in contrast, displayed rapid hyperglycaemia and did not survive the experiment.

It should be noted that there will be a point when the pO_2 within the gill chamber is greater than the pO_2 externally and at this time the animal will effectively be in an anoxic environment. Experimentally a value of 7 torr, as obtained in these experiments may therefore be totally anoxic to the animal.

However, this is an experimental condition to induce a measurable stress on the animal and deoxygenation of this magnitude would rarely, if ever, exist in a natural environment in which the *N. norvegicus* are situated.

An increase of emergence behaviour is an additional physiological adaptation to hypoxia expressed by *Nephrops norvegicus* and this possibly explains an increase of commercial landings, of which a high percentage of animals are either dead or dying, during known periods of hypoxia caused by pollution and eutrophication in summer months (Hagerman and Uglow, 1985). This has been particularly prevalent in the Kattegat. It is suggested that animals which are likely to experience hypoxia are more able to compensate at these times of physiological stress. (For further details see Chapter 1, section 1.1.3.3)

Clearly, this interesting result showing a difference between *Nephrops norvegicus* and *Orconectes limosus,* needs further investigation. It would be necessary to compare the glucose levels of species that are known to be able to withstand long periods of hypoxia, to those of *N. norvegicus.* Alternatively, species that are not tolerant of hypoxia should also be investigated. The burrowing prawn *Calocaris macandreae* for example, is known to experience total anoxia regularly during many weeks, while the results obtained by Taylor and Spicer (1987), demonstrate that *Palaemon elegans* and *P. serratus* clearly show a habitat related glycaemia difference.

The identification of circulating CHH levels, using the previously discussed DAS–ELISA method, could be used to determine what percentage of hyperglycaemia observed is purely the result of CHH. In addition, it would give a greater indication of the stress effect on the animal and elucidate differences between species from differing habitats. For example, it would be expected that a burst of CHH and associated hyperglyceamia, would occur when the animals

are initially subjected to the hypoxic conditions, although during the resulting hypoglycaemia, it would be expected that the levels of CHH would also decline. Results obtained by Keller and Orth (1990) demonstrate that the CHH is only apparent in the haemolymph within the first hour of the stress effect, and that hyperglycaemia only occurs after this first hour and continues for the resulting 10 h. These authors speculate that the resulting hyperglycaemia is not a direct effect of the CHH, but is a more indirect effect due to metabolic changes which require CHH in their early phase. It is, as mentioned above, proposed that the glucose may be quickly metabolised in the first hours, thus preventing a significant build up in the haemolymph. This latter hypothesis would explain the differences observed between *Orconectes limosus*, used in the Keller and Orth study (1990) and *Nephrops norvegicus* used in this study and would support the hypothesis that *N. norvegicus* are able to metabolise glucose more efficiently when there is a limitation of free oxygen. Recent work by Santos and Keller (1992), suggest that hypoxic conditions may stimulate glycolysis and that CHH increases the substrate availability, thus allowing glycolysis to proceed at a higher rate. No increase of lactate levels was observed in destalked animals in normoxic conditions, while CHH injections only cause hyperglycaemia and no lactate increases. It is concluded that in *Carcinus maenas*, used by Santos and Keller, hyperglycaemia due to CHH, cannot be explained on the basis of a decrease of glucose utilisation. Previous experiments, however, demonstrated that during tissue incubation the presence of CHH appears to inhibit glucose uptake by the tissues of the meso-supralittoral crab *Chasmagnathus granulata* (Santos *et al*, 1988). This study suggests that CHH may cause hyperglycaemia by decreasing glucose utilisation. If this explanation holds true for *N. norvegicus*, CHH is only responsible for the initial rise of glucose and over the subsequent 10 h, the hypoglycaemia may be a result of utilisation of glucose by the animal. The measurement of circulating CHH by the use of DAS–ELISA in *N. norvegicus* would help to clarify these results as would the determination of

CHH mRNA levels over a period of hypoxia. It may be possible that a peak of mRNA synthesis could occur either before the peak of CHH, suggesting the direct translation of the mRNA. Alternatively, the mRNA peak may occur later suggesting that a "pool" of mRNA is utilised by the animal under stressed conditions, and that synthesis is stimulated by the exploitation of these reserves.

It is possible to speculate that the initial CHH burst observed in *Orconectes* caused by the hypoxia, may be sufficient to maximally activate the receptor sites in the hepatopancreas and muscles, causing a maximal release of cyclic nucleotides into the system. This may result therefore, in a prolonged release of glucose from glycogen reserves.

Interestingly, the apparent level of the stress imposed on the animal causes varying degrees of CHH release. Anoxia and temperature elevation cause high levels of CHH (120fmol) while starvation and, although not a stress factor, diurnality cause much lower levels of CHH release (40–60fmol) (Keller and Orth, 1990). Correspondingly, the levels of glucose observed in these less chronic stresses (starvation *etc.*) are much lower than those observed in acute stress situations (anoxia, temperature elevation). This may support the above theory concerning the maximal activation of CHH binding sites, but may also lend itself to a further theory. Crustacea may act differently under rapid (anoxia, temperature elevation) or slow (starvation, diurnality, over-crowding, reproduction) CHH release situations, thus creating a fast, high release CHH response and a slow, low release CHH response depending on the circumstances. The occurrence of CHH mRNA levels in the cerebral, thoracic and abdominal ganglia (Tensen, 1991) indicate further sites of synthesis of CHH, other than the MTGXO. Under thermal stress, destalked *Orconectes limosus* have detectable levels of circulating CHH (Keller and Orth, 1990) suggesting that the ganglia, mentioned above, may be able to release hormone under extreme conditions. The pO_2 of water decreases at higher temperatures

and therefore, the CHH release may be due to the temperature and the lower oxygen tensions. It is possible that the release of CHH under varying stressors may be dose dependent. That is, the greater the stress, the greater the release of CHH and hyperglycaemia.

CHH has been shown to stimulate amylase secretion from the midgut gland of *Orconectes limosus* (Sedlmeier, 1988). Both CHH release and amylase production increase glucose levels in the haemolymph, although CHH may regulate glucose levels by the release of amylase rather than by utilising glycogen reserves. During starvation, however, there is an increased secretion of amylase (Fingerman *et al*, 1967). During these periods of stress, CHH may be released to mobilise stored glycogen and would concurrently activate amylase production.

Clearly, much work needs to be carried out in order to understand the role of CHH fully during stress situations. The physiological significance of CHH and stressed induced haemolymph glucose and what if any adaptive role this plays in the Crustacea, needs further clarification. In this present study investigating the role of hypoxia on *Nephrops norvegicus* and in studies by other researchers, such as Keller and Orth (1990), destalked animals did not show a higher mortality compared to that of the intact animals. If eyestalk neuropeptides were physiologically essential for adaptation to such stressors, then one would expect a higher mortality amongst destalked animals than those which remain intact.

The effect of day length on glucose production in *Nephrops norvegicus*, again produced interesting results which, in part, appears to differ from other species investigated. Hamann (1974) demonstrated the occurrence of diurnal fluctuations of glucose in *Orconectes limosus*, with a maximal nocturnal peak of haemolymph sugar. It was established that sinus gland extirpation caused a decrease of circulating glucose and in addition removed the diurnality. Results

obtained by Gorgels–Kallen and Voorter (1985) demonstrated that this circadian rhythm exists in other species, the crayfish, *Astacus leptodactylus*, and circulating CHH rhythmicity was detected over a light/dark period, using DAS–ELISA (Kallen *et al*, 1990). Keller and Orth (1990) demonstrated that the glucose rhythm in *O. limosus* corresponds in a similar rhythm of CHH, with a peak of both during the dark period, using RIA. More over, Kallen *et al* (1988) demonstrated that the endogenous diurnal rhythm of glucose, giving a maximal peak during the dark period, could be shifted out of phase by altering the light/dark period. These results demonstrated that the increase of CHH proceeds the occurrence of hyperglycaemia in the crayfish. A diurnal rhythm of CHH mRNA has been identified in *O. limosus* by Tensen (1991), which identifies a maximal level of mRNA at the peak of the light period, some 4 h prior to the increased release of CHH at the start of the dark period. During release of CHH and subsequent hyperglycaemia, there is a decrease in synthetic activity of the CHH producing cells. The whole process is repeated, to a lesser extent, in the early morning period, resulting with CHH release just before the start of the light period. These data suggest that CHH release is directly related to mRNA expression (Tensen, 1991).

From initial investigations reported here, the circadian rhythm of glucose in *Nephrops norvegicus*, does not appear to be as obvious as in other species investigated. The eyes of *N. norvegicus* are relatively large when compared to those of other genera within the Nephropidae. Their superposition eye is characteristic of nocturnal crustaceans and it is clear therefore that the *N. norvegicus* eye is adapted to use in low light intensities (Aréchiga and Atkinson, 1975; Loew, 1976; Chapman, 1980). Dark and light adaptation in crustacean compound eye, occurs by the movements of absorbing and reflecting pigmentary material. The migration of pigment is directly affected by a change of light intensity, by circadian rhythms and in some cases, by neurosecretion (Rao,

1985). The speed of this adaptation is directly proportional to the depth of water in which these crustaceans are found, however, it has been demonstrated that in *N. norvegicus*, only the rate of dark adaptation and not light adaptation, is affected by circadian rhythms (Shelton *et al*, 1986). As *N. norvegicus* is prone to light–induced irreversible retinula cell breakdown when the eye is exposed to daylight (Loew, 1976; Shelton *et al*, 1985), the dark adaptation of the eye of *Nephrops* could be of some adaptive value (Shelton *et al*, 1986). Dark adapted animals exposed to daylight, would light adapt quickly, thus protecting the retinal pigments. Alternatively, light adapted animals, if exposed to temporarily reduced light intensities during the day, should not dark adapt, as this would make the animal susceptible to sudden increases of light intensity. It has been identified that dark adapted animals are much more susceptible to retinal damage that light adapted animals and in addition, *N. norvegicus* raised from very deep water, where little light penetrates, may have permanently dark adapted eyes. After prolonged exposure (2.5 h) to high surface light intensities, the visual pigment becomes irreversibly bleached and the structure of the rhabdomes breaks down (Loew, 1976). Although a short ten minute burst of light does not appear to damage the eye (Chapman, 1980), it is considered that maintenance of animals in bright light will cause blindness, although it is not clear if light induced blindness is permanent. It is possible that deeper caught, permanently dark adapted *Nephrops*, will have the retinal pigment damaged quicker, than shallower caught animals.

As previously discussed in Chapter 1, the effect of light intensity and burrow emergence has been investigated in some detail (for review see Chapman, 1980). *Nephrops norvegicus* remain in their burrows for the majority of each 24 h period. Data obtained by underwater television (Chapman and Howard, 1979) and from trawling results (Farmer; 1974a; Aréchiga and Atkinson, 1975; Atkinson and Naylor, 1976) indicate that burrow emergence is

directly related to light intensity. In water less than 20m, peak burrow emergence and activity is around midnight, while at depths in excess of 150m, it occurs mainly around midday. Clearly, this pattern is not fixed, as much depends on the season, the tides and tidal flow, turbidity of the water and even the size of the animals. Work by Anderson (1962) suggest that *N. norvegicus* become less responsive to light with increasing size. Experiments suggest that light intensity is the single most important trigger for emergence activity. Over most of its depth range, *N. norvegicus* emerges during the period of rapidly changing light intensity at dawn and dusk (but still within its optimal range). Emergence may be induced by a decrease (at dusk) and an increase (at dawn) in light intensity. In deep water, only an increase of light intensity would be effective (at midday) to induce emergence, a decrease in light intensity in shallow water, would induce crepuscular and nocturnal emergence (Chapman, 1980).

The experiment summarised in figure 5.1 as described earlier, demonstrated two groups of animals, one showing apparently rhythmic levels of glucose and another displaying no obvious rhythmicity. As *Nephrops norvegicus* were obtained commercially, there were few details on the maintenance of animals on the boat following capture and therefore, it is possible that retinal damage had occurred in experimental animals. Once in the laboratory, animals were kept in partially covered tanks in order that the lights of the aquarium did not cause further damage. This damage to the retinal pigment may explain why only some of the animals showed any rhythm of haemolymph glucose. Furthermore, *N. norvegicus* are trawled commercially over depths ranging from 20–160m, therefore, it is conceivable that our sample of animals contains both shallow water, light adapted animals, displaying a dark phase burrow emergence pattern and deep water, dark adapted animals showing a light phase burrow emergence. This may account for the group of animals showing a glucose rhythm, these being from shallower water that respond to the change

of light intensity at dawn and dusk. However, if the other arhythmic group were deep water animals, it would be expected that their peak of glucose would occur at midday. In actuality, there is no peak of glucose, suggesting that either, in deep water animals there is no endogenous rhythm of glucose, or that these animal were permanently dark adapted and that the retinal pigments were damaged, thus preventing the detection of photoperiod. It is conceivable that these could be shallow water animals that have had retinal pigment damage.

Future experiments, should be carried out on animals obtained from differing depths and care should be taken during aquarium maintenance, in order to protect the retinal pigment from damage. A red light when shone into the eye of *Nephrops norvegicus*, will emit a golden glow if the pigments are not damaged. This simple test will ensure that all test animals are able to detect photoperiodic changes. Further, the measurement of the release of CHH into the haemolymph and the identification of peaks of mRNA production, would greatly enhance our understanding of this area of study. Clearly, the picture is unclear and with the relatively small sample size used it would be necessary to obtain further results in order to obtain a better understanding of controlled rhythms in this species.

Chapter 6.

The detection of *Nephrops norvegicus* CHH using molecular biological techniques.

6.1 Introduction.

The polymerase chain reaction (PCR) allows the *in vitro* sequence specific amplification of target DNA from a double stranded DNA template. The method is capable of the detection and amplification of a single gene copy. The PCR has been applied to many areas of biological research, including recently, crustacean endocrinology. The PCR consists of a number of cycles, during which, two oligonucleotide primers; a 5' sence and 3' antisence, flank the DNA segment to be amplified. The synthesis and amplification proceeds between the two primers. The DNA is strand separated at approximately 94°C, typically for 1 minute and the primers are then annealed to the target DNA by cooling to between 40–60°C for 1 minute. This is followed by primer extension at up to 72°C for at least 1 minute for each cycle. Typically, between 25 and 45 cycles will be performed in order to amplify sufficient product. Often a "hot start" is performed to improve the specificity of the reaction. The template is denatured for several minutes at 90°C and the reaction cooled to 80°C before addition of the DNA polymerase. After the final cycle, the reaction is maintained at the optimal temperature of *Taq;* 72°C for 10 minutes to ensure that all the amplified DNA is double stranded. As the name suggests, the PCR allows the amplification of the DNA to increase exponentially as the number of cycles increase. The PCR product can be directly cloned into a plasmid or M13 vector and recombinants transformed to and propagated in *E. coli.* The nucleotide sequence can then be determined (Bej *et al,* 1991 for review).

The complete sequence of two structurally different hyperglycaemic neuropeptides have been deduced by the cloning of PCR amplified cDNA encoding CHH of *Homarus americanus* by the use of oligonucleotide primers based on partial amino acid sequence of CHH (Tensen, 1991; Tensen *et al,* 1991a; 1991b). This technique enabled the sequence of CHH A and CHH B to

be determined, the cDNA of the former encoding a peptide of 8535Da and the latter encoding a peptide of 8638Da. Results obtained using FAB/MS HPLC purified fractions of CHH II (A) and for CHH VII (B) indicated a molecular masses of 8578Da and 8656 ±25Da respectively (Tensen, 1991; Tensen *et al*, 1991c), while Soyez *et al* (1990), deduced molecular masses of 8577Da and 8633Da for four basic CHH isoforms in the same species. The comparison of sequences of CHH isoforms of ***Homarus americanus*** obtained by molecular techniques and their relative homologies to *Cam* CHH, *Hoa* and *Cam* MIH's and *Hoa* VIH are reviewed by Tensen (1991) and Keller (1992).

The cloning and sequencing of cDNA of eyestalk neuropeptides and the identification, distribution and quantification of specific mRNA's in various crustacean tissues has now become possible following the publication of their amino acid sequences. The mRNA's of CHH and GIH have been localised in the X-organ sinus gland complex of ***Homarus americanus*** (De Klein *et al*, 1992). In addition, the CHH precursor, pre-pro CHH, has been sequenced from the shore crab, ***Carcinus maenas*** (Weidemann *et al*, 1989) and two different CHH precursors from the same species, ***Orconectes limosus*** pre-pro CHH A and *O. limosus* pre-pro CHH B have been identified and sequenced using molecular biological techniques. These contain the reported CHH amino acid sequence (Kegel *et al*, 1991), preceded by a signal peptide of 26 amino acid and a CHH precursor related peptide (CPRP) (Tensen *et al*, 1991d) of 33 amino acids. The CPRP of *O. limosus* and *H. americanus* has been recognised as "peptide H" of the land crab, *Cardisoma carniflex* (Newcomb, 1987) and "peptide C" that flanks CHH N-terminally within the prohormone of *C. maenas* (Weidemann *et al*, 1989). CPRP is analogous to APRP (Adipo kinetic hormone Precursor Related Peptide) (Hekimi *et al*, 1989) and although its role of CPRP is not fully understood, numerous theories for its function have been postulated (Weidemann *et al*, 1989; Tensen 1991; Tensen *et al*, 1991d).

Degenerated oligonucleotide primers, based on the amino acid sequence of the MIH of *Carcinus maenas* (Webster, 1991), have been used in the PCR and resulting cDNA has been cloned and a sequence for MIH obtained (Klein *et al*, 1992). Work to sequence the complete MIH precursor and the localisation of MIH mRNA is currently under investigation by the same authors. *In situ* hybridisation to detect CHH mRNA in the X-organ of peneid shrimps, is also being researched (Laverdure *et al*, 1992a), while the same technique has been used to detect mRNA encoding GIH in the eyestalk of *Homarus* (Laverdure, 1992b).

The aim of this chapter was to detect *Nephrops norvegicus* CHH cDNA by the PCR using oligonucleotide primers complimentary to regions of the CHH sequence from the lobster, *Homarus americanus*. The detection of CHH production by immunological and molecular biological techniques using PCR generated cDNA encoding CHH, has been demonstrated to be species non-specific within the Astacidea (Tensen, 1991). The PCR product would ultimately be cloned into a vector, transformed into *E. coli* and a nucleotide sequence obtained. Comparisons with known sequences of could then be made.

6.2 Materials and methods.

6.2.1 Cloning of cDNA.

Full details of the procedures for the cloning of CHH cDNA from *Nephrops norvegicus* are described in Chapter 2. Total RNA of the whole optic ganglia was prepared from freshly sacrificed *N. norvegicus* by a modified guanidinium thiocyanate procedure (Maniatis *et al*, 1982). The product was reverse transcribed by reverse transcriptase II (BRL) to produce 1st strand cDNA, the details of which are described in Chapter 2. Amplification of cDNA

using pairs of oligonucleotide primers based on the sequence of the lobster, *Homarus americanus*, a gift from Professor F. Van Herp (Nijmegen), complimentary to regions of the CHH sequence were used in the Polymerase Chain Reactions (PCR).

Primers coding nucleotides 1-23, 64-86 and 202-218 (P1, P2 & P3) for the *Homarus americanus* CHH were used in the PCR (Tensen, 1991; Tensen *et al*, 1991b). 35 cycles of PCR amplification with primers 1 and 2 was adopted with an annealing temperature of 45°C while primers 1 and 3 were used in a 40 cycle PCR amplification with an optimised annealing temperature of 47°C.

The PCR product was resolved by electrophoresis on a 3.5% tris-borate-EDTA buffered agarose (Nusieve) gel stained with ethidium bromide. The product size was compared with known standards and its concentration from the gel was estimated and removed from the agarose using a Spinex column. The PCR product was used in the ligation reaction which is detailed in Chapter 2, and was incubated for 16 hours at 12°C. The T4 DNA ligase was inactivated at 65°C for ten minutes. The cells were transformed with 100ng of ligated vector following the Invitrogen TA cloning kit protocol.

6.3 Results.

6.3.1 Preliminary cDNA Cloning of CHH from *Nephrops. norvegicus.*

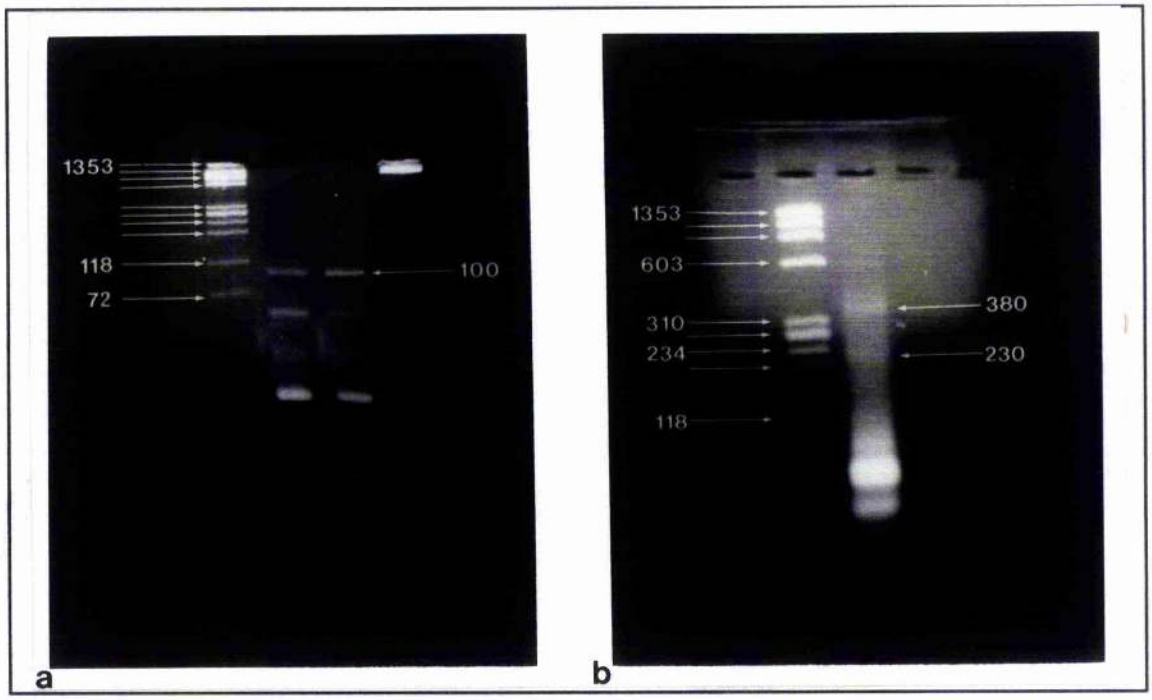
Amplification of cDNA between primers 1 and 2 and primers 1 and 3 on a 3.5% tris-borate-EDTA agarose (Nusieve) gel stained with ethidium bromide showed that primers 1 and 2 produced a strong signal corresponding to a 100bp product (figure 6.1a). A weaker signal was obtained by using primers 1 and 3

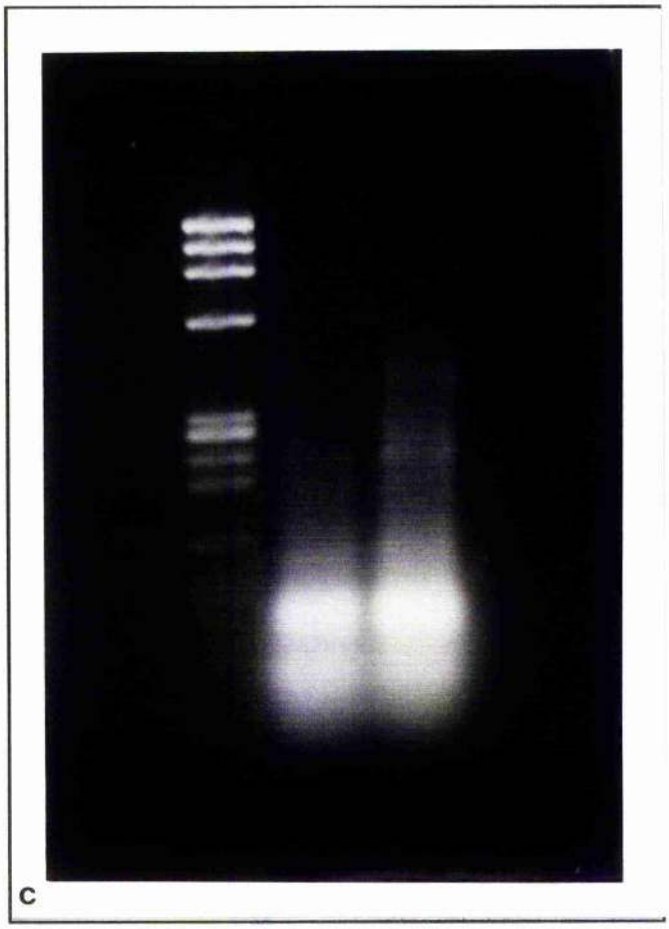
Figure 6.1a and 6.1b.

Detail of a 3.5% tris-borate-EDTA buffered agarose Nusieve gel stained with ethidium bromide showing results of the PCR of amplified cDNA obtained from total RNA of *Nephrops* eyestalks. Use of *Homarus* oligonucleotide primers 1&2 for 35 cycles at an annealing temperature of 45°C show a product of 100 bp (figure 6.1a), and *Homarus* primers 1&3 for 40 cycles at 47°C a product of 230 bp (figure 6.1b). An additional band of 380 bp was obtained with primers 1&3 and this is thought to be CHH B-RI (see text for discussion). Known standards (Ø x 174/Hae III) are shown in the left hand lanes. These are of 72, 118, 194, 234, 271&281 (which run as a pair), 310, 603, 872, 1078 and 1353 base pairs.

Figure 6.1c

Detail of a 3.5% tris-borate-EDTA buffered agarose Nusieve gel stained with ethidium bromide showing results of the PCR of amplified cDNA obtained from total RNA of *Nephrops* eyestalks. Use of *Homarus* oligonucleotide primers 1&3 for 40 cycles at an annealing temperature of 43°C (middle lane), and *Homarus* primers 1&3 for 40 cycles at 45°C (right lane). Known standards (Ø x 174/Hae III) are shown in the left lane. These are of 72, 118, 194, 234, 271&281 (which run as a pair), 310, 603, 872, 1078 and 1353 base pairs.


1353
118
72
100
a
1353
603
310
234
118
380
230
b


c

which gave a 230bp product with an additional amplification product sized at approximately 380bp (figure 6.1b). Figure 6.1c show the results of the PCR using primers 1 and 3 at differing annealing temperatures carried out at 43°C and 45°C respectively, in order to optimise the reaction. In the left hand lane on each gel, DNA size markers are shown, making an estimation of the cDNA products possible. The ligation of the PCR product was not successful.

6.4 Discussion.

The most critical temperatures of the PCR are the denaturation and reannealing temperatures. The denaturing of the double stranded DNA to a single strand, allows the amplification process to take place, while the annealing temperature determines the specificity of the PCR. Too low a temperature causes miss priming and a temperature that is too high prevents the proper annealing of the primers and hence no amplification. The results observed in this chapter demonstrated that, depending on the temperature at which the annealing took place for the PCR between primers 1 and 3, there may be variation in the banding obtained (figures 6.1b; 6.1c; 6.1d).

PCR showed that primers specific for the lobster CHH, probably have a high degree of homology with the same regions of the *Nephrops norvegicus* CHH molecule. A strong signal was observed with primers 1 and 2 indicating a 100bp product, although a weaker signal with greater variety of banding was seen using primers 1 and 3. This may indicate that possible differences in the nucleotide sequence of *Homarus* CHH and *Nephrops* CHH may lie at the 3', carboxy end. Part of this additional banding may be a 380bp product described by Tensen (1991) as CHH B-Retained Intron (CHH B-RI). It is suggested that

this product results from partially or unspliced CHH B encoding pre-mRNAs. Alternatively, the 380bp product described above, could be an artifact product. Due to the amplification properties of the PCR, accidental contamination of samples with exogenous DNA, will result in the production of artifact products, thus, the fidelity of PCR should be kept in mind.

The use of total RNA from *Nephrops norvegicus* optic ganglia and subsequent cDNA amplification by the use of the PCR described above, indicates that the partial N-terminal sequence obtained in Chapter 4 is almost certainly not that of *Nen*–CHH. The use of oligonucleotide primers specific to the 5' N-terminus and the 3' C-terminus of *Homarus americanus* in the above PCR and the use of antisera raised in both *Orconectes limosus* and *Homarus americanus* (see Chapters 4 and 7), have demonstrated that there is probably considerable homology of the CHH sequence between *Nephrops norvegicus* and the above two species. The partial sequence described in Chapter 4 has little homology to the *Hoa* CHH A or *Hoa* CHH B sequences that were successfully deduced using the 30 mer primer 1 (Tensen, 1991; Tensen *et al*, 1991b), the same primer used in this study.

The inability to clone and sequence the PCR product obtained in this study is mainly the result of the low yield of product acquired from the PCR reaction. The result of this was that the high purity and sufficiently high concentration of DNA that is required for the success of the ligation reactions, could not be obtained. The reaction proved to be difficult to reproduce, probably because of the degradation of RNA template for cDNA synthesis, which in turn is due more to the difficulty of RNA isolation from the tissue used.

The use of *Nephrops norvegicus* optic ganglia for RNA purification, yielded low quantities of total RNA and similar low yields were observed during total RNA extraction of the eggs of *N. norvegicus* (see Chapter 7). Indeed,

attempts to obtain poly A^+ RNA, by the application of the total RNA to an oligo dT column, were unsuccessful. The tissues of *N. norvegicus* are not particularly suitable for RNA preparation. Conventional protocols, described above and in Chapter 2, did not produce the yields and purity of RNA routinely expected from mammalian and teleost tissues.

Chapter 7.

Studies on CHH and GIH during embryonic development of *Nephrops norvegicus*.

7.1 Introduction.

Since the report that eyestalk removal from the mature shrimp, *Palaemon serratus*, led to accelerated ovarian growth (Panouse, 1946; see also Adiyodi and Adiyodi, 1970 for review), much work has been carried out with the specific aim to stimulate ovarian growth and ultimately egg production, in commercial crustacean species. Research appears to be divided into two groups; the first being ovarian development and the endocrine control of vitellogenesis and the second being the endocrine control of development of larvae and post larval crustaceans (see Adiyodi and Adiyodi, 1970; Schroeder, 1987; Charniaux-Cotton and Payen, 1988; Keller and Sedlmeier, 1988 for reviews).

The role of eyestalk neuropeptides in particular, and their site of synthesis and release in adult crustaceans, has been studied intensively for a number of years. In contrast, little work has been carried out on larval or embryonic neuropeptide production. Work by Gorgels-Kallen and Meij (1985) demonstrated immunocytochemically the CHH producing system in the eyestalks of *Astacus leptodactylus* during larval and post larval development. The MIH neurosecretory system in *Carcinus maenas* larvae has been described using specific antisera (Webster and Dircksen, 1991). The primary structure of GIH has been identified in the lobster, *Homarus americanus* (Soyez *et al* 1991) and has been characterised in the crayfish, *Procambarus bouvieri* (Huberman *et al*, 1992). GIH is a peptide of 77 amino acid residues, that has been shown to inhibit ovarian development (Bomirski *et al*, 1981; Soyez *et al*, 1987) and more precisely vitellogenin endocytosis (Jugan and Soyez, 1985). A stimulatory effect of a sinus gland peptide on the development of oocytes has been investigated by a number of researchers (Van Deijnen, 1986; Soyez *et al*, 1987; Van Herp, 1988; Tensen *et al*, 1989). A vitellogenin binding protein has been identified on the membrane of the egg of *Orconectes limosus* (Jugan and Van

Herp, 1989), however, it is thought that this is the only crustacean eyestalk neuroendocrine study that has been carried out on the eggs. It is speculated that GIH may affect the distribution and the binding capabilities of the oocyte vitellogenin receptor (Jugan and Van Herp, 1989). The role of ecdysteroids during embryonic development have been recently investigated (McCarthy and Skinner, 1979; Chaix and DeReggi, 1982; Lachaise and Hoffmann, 1982; Spindler *et al*, 1987), in particular the role of high ecdysteroid titres and their association with the secretion of the embryonic envelope (Goudeau *et al*, 1990) and the profile and composition of embryonic ecdysteroids during oogenesis of the fresh water prawn, *Macrobrachium rosenbergii* (Young *et al*, 1991).

Recent work by Rotllant *et al* (1991; 1992) identified the localisation of CHH and GIH in the eyestalk of larval and post larval *Homarus gammarus*, using specific antisera and *in situ* hybridisation. *H. gammarus* had already been shown to be neuroendocrinologically functional at stage I (Rotllant *et al* , 1991). Approximately 20 CHH and 20 GIH immunopositive cells were identified, although occasionally both CHH and GIH immunoreactions were observed in the same cells. The synthesis and storage of both neuropeptides in the eyestalks of *Homarus gammarus*, therefore, occurs in stage I larvae (Rotllant *et al*, 1992).

The detection and localisation of CHH mRNA with non-radioactive *in situ* hybridisation in the eyestalks of *Orconectes limosus* and *Homarus americanus* in combination with immunocytochemical techniques have demonstrated the colocalisation of CHH in identical CHH cells (Tensen, 1991; Tensen *et al*, 1991a; Van Herp and Kallen, 1991). The use of PCR with oligonucleotide probes have been used to identify CHH mRNA in the medulla terminalis and various other tissues from *Orconectes limosus* (Tensen, 1991; Tensen *et al*, 1991a) including the cerebral, thoracic and abdominal ganglia. The use of similar molecular biological techniques have been utilised to identify the variation of mRNA encoding CHH during a light/dark cycle in the crayfish

Orconectes limosus (Tensen 1991). The dual detection of CHH production in various tissues by immunological and molecular biological techniques using PCR generated cDNA encoding CHH and antisera has been demonstrated to be species non-specific within the Astacidea.

Research initiated in this chapter investigated a number of aspects of the embryonic development of *Nephrops norvegicus*. First, the culture of *N. norvegicus*, and the development of growth curves and morphological and behavioural descriptions during egg development. Secondly, the use of ELISA, using CHH and GIH specific antisera, on various stages of egg maturation was carried out in order to detect at which stage of embryonic development the two neuropeptides could be detected within the egg. Thirdly, it was hoped to detect CHH cDNA by PCR using the degenerated oligonucleotide primers discussed in Chapter 2 and Chapter 6 of this thesis, thus indicating the synthesis of CHH by the embryo.

The anti-*Orconectes limosus* CHH and the anti-*Homarus americanus* GIH were gifts from Professor R. Keller, Bonn and from Dr D. Soyez, Paris, respectively. The degenerated oligonucleotide primers, that were used in the PCR, were a gift from Professor F. Van Herp, Nijmegen.

7.2 Materials and Methods.

7.2.1 Rearing and development of eggs from *Nephrops norvegicus*.

Female berried *Nephrops* were obtained from mid September until March and were maintained as discussed in Chapter 2, section 2.2. Eggs were removed from the female and reared by the method described in Chapter 2. Ten live eggs were examined every two to three days. After a month the eggs were

assessed weekly. Once the heart was visible, its beat was confirmed for each embryo before measurements were made. Three measurements were made using an eye piece graticule across the diameter of the egg in order to obtain an accurate average value of egg size increase. The mean diameter for each egg was calculated and the total mean for each group of ten eggs was then determined. Measurements of the length and width of the developing eye was made, as this can be used as an assessment of embryo development (Perkins, 1972) and a mean value was obtained for each group of eggs measured. The Perkins eye index is defined as the average length and width of the brown screening pigment spot (measured in micrometers) in the lateral eyes (Perkins, 1972). Prior to the appearance of eye pigment, time was used as characterising development. However, the stage of appearance of developmental events is expressed as percentage development of total embryogenesis (Helluy and Beltz, 1991). These authors have demonstrated that there is little difference during early embryogenesis between staging based on time and that based on eye index. Once eye pigmentation was observed in the developing embryo, the eye index (EI) was used as a morphometric marker. A description of the eggs was also made as visual and behavioural changes are an important indication of development.

7.2.2 Preparation of total RNA from eggs of *Nephrops norvegicus.*

In order to obtain total RNA from the eggs of *Nephrops*, the method used for the preparation of total eyestalk RNA was attempted. The materials and methods for these techniques are described in Chapter 2. Approximately 1g of *Nephrops* eggs, at about 50% development (Helluy and Beltz, 1991)(see section 7.2.1 and Table 7.1 for details of developmental staging), were washed with DEP water and placed into a centrifuge tube containing guanidinium

thiocyanate/β-mercaptoethanol (15.3ml guanidinium buffer + 1.7ml β-mercaptoethanol). Once homogenised 6M lithium chloride (LiCl) was added (23mls to each 17mls of homogenate) and mixed using a 10ml syringe and blunt 18G needle in order to shear the high molecular weight DNA. The amount of precipitated DNA/RNA was visibly much greater than that precipitated when using medulla terminalis as a starting material. This was transferred to a 50ml poly-propylene centrifuge tube and allowed to precipitate overnight at 4°C.

Following centrifugation at 10,000g for 90 minutes at 4°C, the supernatant was discarded and it was then attempted to raise the pellet in 3M LiCl. However, the pellet had become totally solid and could not be resuspended. It was presumed that the large amount of protein, carbohydrate and lipid within the eggs, had resulted in an insoluble precipitate following LiCl treatment. When the same protocol was adopted using a reduced number of eggs as starting material, the yield of RNA was extremely poor. An alternative method was utilised and is summarised briefly below. The details of this method are described in Chapter 2 and is a modified method described by Sambrook *et al* (1989).

Approximately 1g of *Nephrops* eggs, at about 50% development (Helluy and Beltz, 1991)(see section 7.2.1 and Table 7.1 for details of developmental staging), were homogenised by the method described in Chapter 2. Following two extractions with phenol:chloroform:isoamyl alcohol and centrifugation, the aqueous phases were then combined and the RNA and DNA precipitated by the addition of 3M sodium acetate and ethanol for 2 hours on ice. Following centrifugation the dried pellet was mixed with 8M LiCl and stored at -20°C overnight.

The RNA was recovered by centrifugation and the pellet was washed in ice cold 70% ethanol, recentrifuged for 10 minutes, the supernatant discarded

and the pellet vacuum dried at room temperature. The pellet was a deep purple colour which was possibly due to contamination with a natural dye. It was resuspended in 1ml of DEP water, reprecipitated with 3 volumes of ice cold absolute ethanol and stored at -20°C. The RNA yield was quantified spectrophtometrically at 260:280nm as described in Chapter 2 and although the colour appeared not to effect the RNA quantification, it was considered that it may effect cDNA synthesis and therefore attempts to remove the colour were made using a Qiagen column.

The support medium of the Qiagen column contains a hydrophobic anion exchange resin, which actively binds nucleic acids and prevents the non-specific binding of other substances. Once the RNA sample was bound to the support medium, it was hoped that by altering the molarity of the salt eluent, it would be possible to separate the contaminating coloured dye from the total RNA of the eggs. The protocol is described below.

4mls of 2m NaCl and 250mM MOPS, pH7.0, was added to the redissolved pellet, obtained by the procedure described above. The Qiagen–tip 500, was equilibrated with 10mls of buffer containing, 400mM NaCl, 50mM MOPS, 15% ethanol, 0.15% Triton X - 100, at pH7.0, and allowed to empty by gravity flow. The sample was applied to the Qiagen column and allowed to enter the resin by gravity flow. The column with the bound sample was then washed with 30mls of 400mM NaCl, 50mM MOPS and 15% ethanol, pH7.0. In order to elute the RNA, the column was washed with 900mM NaCl, 50mM MOPS, 15% ethanol, and 6M urea, pH7.0; the urea was added just prior to use. The RNA was precipitated from the eluate with 1 volume of isopropanol for 10 minutes on ice and centrifuged at 15,000g for 30 minutes at 4°C. The pellet was washed in ice cold 70% ethanol, dried and resuspended in 1ml of DEP water and the RNA yield was quantified at 260:280nm as described in Chapter 2.

The methodology of the cDNA synthesis from egg total RNA and the amplification of the cDNA by PCR has been described in detail in Chapter 2 and will not be described here.

7.2.3 The use of ELISA on various stages of egg development.

Eggs of *Nephrops* were raised by the method described in Chapter 2 and groups of 20 ova were collected and estimated to be at 12%, 24%, 50% and 90% development (Helluy and Beltz, 1991)(see section 7.2.1 and Table 7.1 for details of developmental staging). Each group of eggs was bathed in 1ml of 0.1M phosphate buffered saline (PBS), the capsule of each was ruptured manually, and then homogenised ultrasonically on ice (MSE Soniprep 150 homogeniser) for two 30 second bursts. The resulting homogenate was centrifuged at 15,600g for 20 minutes (IEC Centra-M microcentrifuge) and the supernatant was collected. The supernatant was then applied to a 96 well microtitre plate (Greiner Labortechnik, ELISA) and a direct ELISA using both the anti-*Orconectes* CHH and anti-*Homarus* GIH antisera, was carried out as described in Chapter 2.

A separate group of 20 eggs was used for each antibody, with the antigen binding taking place on different plates for each antibody used. All samples were carried out in triplicate. A dilution gradient of the CHH active fraction 42 (see Chapter 4) was used as a positive control, while PBS was used as a negative control.

7.3 Results.

7.3.1 Rearing and development of eggs from *Nephrops norvegicus.*

The sequence of developmental and morphometric data obtained during the embryogenesis of *Nephrops norvegicus*, is presented as EI (Eye Index) measurements and mean egg diameter measurements. The stage of appearance of development is expressed as percentage development of total embryogenesis and this is compared to the EI data. If the development of the eggs of *N. norvegicus* follow a similar development to that of *Homarus americanus* (Helluy and Beltz, 1991), then the experimental brood of *N. norvegicus* eggs appeared initially to be at E6%, estimated as day 10 following extrusion.

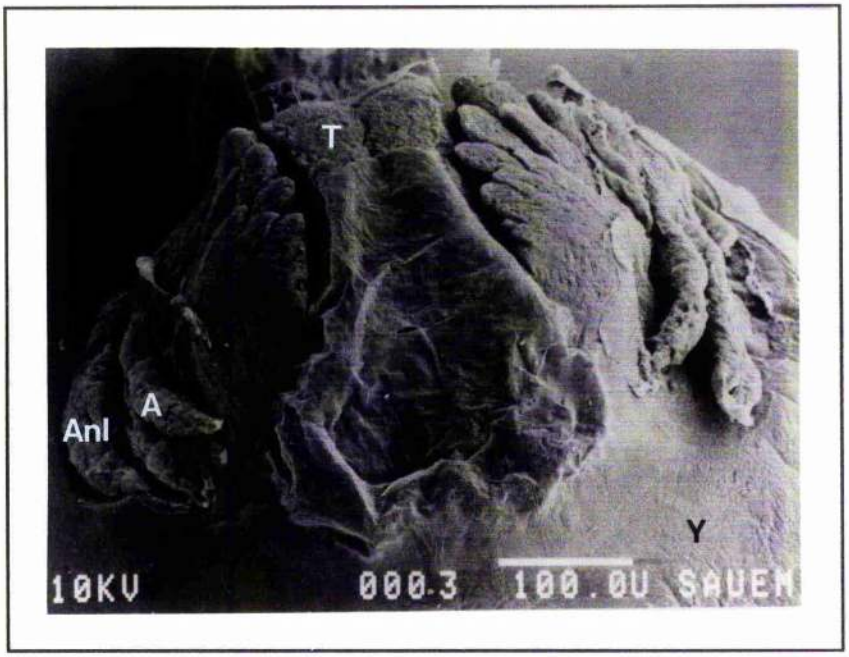
At E6%, the egg is a dark green and granular in appearance and contains large numbers of small yolk globules evenly distributed throughout the entire egg. The optic lobes appear as a white cloud of cells and throracoabdominal processes are clearly outlined. There is a retraction of the yolk mass from the egg wall, so that the dark green yolk occupies 95-98% of the egg volume. Dissection reveals little consolidation of the cells and although there are early developmental appendages, they cannot be easily differentiated by light microscopy. Scanning electron microscope (SEM) investigations, however, clearly show the formation of embryonic limb differentiation (figure 7.1). By E8%, there is a slight change of colour, with 50% of the egg observed to be a lighter green than the initial dark green. The development of nauplius appendages are visible, with the formation of the abdomen along the axis of the egg from the thoracic appendages towards the cloud cells. The abdominal reflexion can only be seen under SEM, and the beginnings of telson differentiation is also visible at this stage. The yolk occupies approximately 95% of the egg's volume at E8%.

Figure 7.1.

SEM photograph of a dissected embryo of *Nephrops norvegicus* at approximately E6%, showing details of development of the embryonic antennula, antenna, maxillipeds and telson. The telson can be seen as a single fused structure as are the maxillipeds. 95% of the volume of the egg is made up of the dark green yolk. The antennae are beginning to show the first signs of a biramous structure. Abbreviations: *Anl antennula*, *An* antenna, *M* maxilliped, T telson, *Y* yolk. Photograph courtesy of R. Clifford.

Figure 7.2.

SEM photograph of a dissected embryo of *Nephrops norvegicus* at approximately E10–11%. The differentiation of embryonic limbs and telson are clearly visible, the telson itself is beginning to part. The embryonic eye is begginning to develop. Abbreviations: *T* telson, *Anl antennula*, *An* antenna, *M* maxilliped. Photograph courtesy of R. Clifford.


T
A
Anl
Y
10KV
000.3
100.0U SAUEM


T
A
M
Anl
10KV
0004

At E10-11%, the yolk still occupies the same volume within the egg, however, the appendages are more clearly defined, with the appearance of segmentation in the thoracic limbs. The abdominal reflexion has occurred to the point that the formation of the telson can be just be observed, although this is only clearly visible after SEM investigation (figure 7.2). By E12-13%, pigment can be seen as a small crescent of dark cells in the same region as the cloud cells (figure 7.3). There is considerable cytoplasmic streaming occurring in the region of the appendages, which can now be defined into approximately four pairs. There is occasional muscular twitching. The EI was measured at 107μm. By E15%, approximately half the embryos had a heart beat and this was now identified in each egg prior to its measurement. A descriptive summary of the developmental stages is provided in Table 7.1.

Reproductive development of the egg cohort described above, was arrested after E15%. A second cohort was obtained the following September and morphological and developmental changes were monitored by Miss Rachael Clifford, an honours student of Dr. M.G. Bentley. At various stages of development, eggs were collected from this cohort in order to test for immunoactivity to the CHH and GIH antisera (see section 7.2.3). The developmental stages of the eggs which were used are summerised in table 7.1 and photographs with legends described as figures 7.3, 7.4, 7.5 and 7.6. This second cohort was continued up to the first zoeal stage (figure 7.7), however, none of the larvae survived until the second zoeal stage.

Table 7.1. Description of developmental stages of *Nephrops norvegicus* eggs used for ELISA and total RNA preparation.

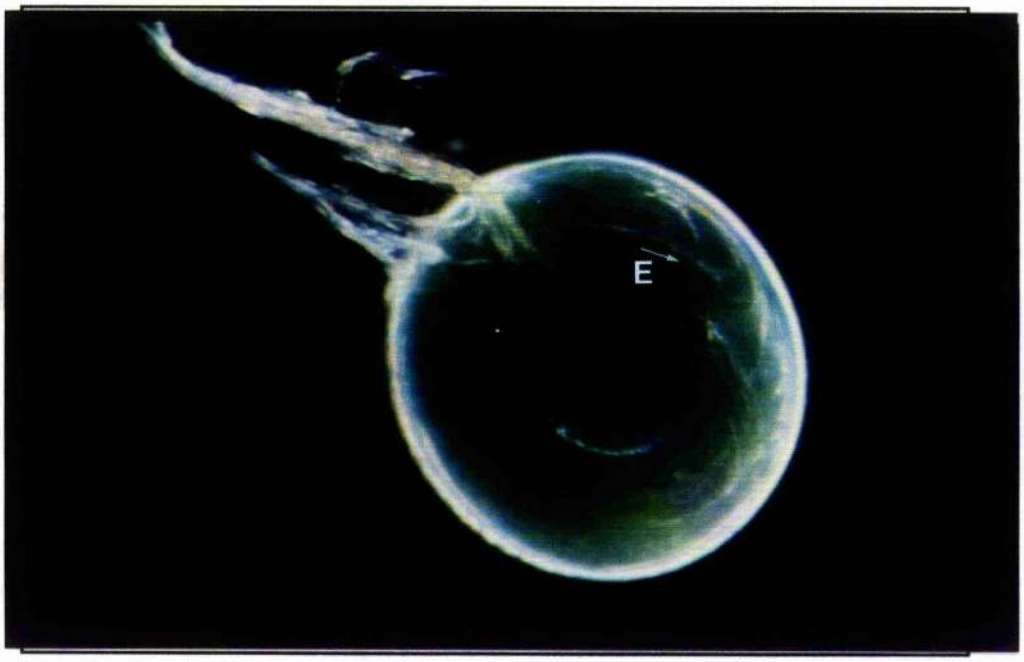
Percentage Development (by Perkins Eye Index)	Egg Colour	Embryonic Phase	Embryonic Description
12%	Dark Green	Naupliar phase	First eye pigment and occasional twitching observed, cytoplasmic streaming, no heart beat visible.
24%	Light green	Early metanaupliar phase	Chromatophore pigment present in some limbs, heart beat present in all eggs.
50%	Green/yellow	Mid-metanaupliar phase	Clear tail flips after removal of egg envelopes, eyes large and oval with formation of ommatidia, clear limb development and segmentation observed under light microscopy, large number of chromatophores.
90%	Yellow/orange	Late metanaupliar phase	Embryo almost fully developed, limbs fully differentiated, total relexion of abdomen.

Figure 7.3.

Unfixed, intact egg of *Nephrops norvegicus* at approximately E12%. The eye pigment is just visible as a small crescent (EI was measured at 107μm). The dark green granular yolk occupies approximately 95% of the egg volume. Abbreviation: *E* eye. Photograph courtesy of R. Clifford.

Figure 7.4.

Unfixed, intact egg of *Nephrops norvegicus* at approximately E24%. The eye pigment has become darker and more consolidated. The ommatidia are beginning to form and the eye has is become ovoid in shape. The heart beat is visible and the yolk volume is being reduced and forming two separate caeca. A few chromatophores begin to form (not seen in photograph) Abbreviation: *E* eye. Photograph courtesy of R. Clifford.


E

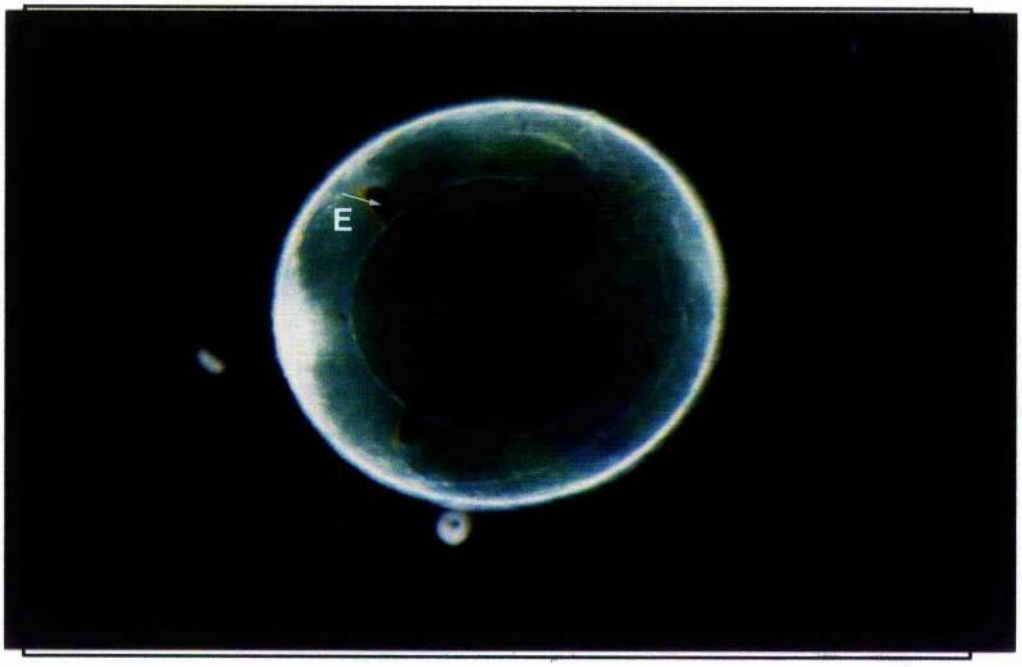

E

Figure 7.5.

Unfixed, intact egg of *Nephrops norvegicus* at approximately E50%. The eye is now ovoid and has clearly defined ommatidia. The thoracic appendages can now be seen to contain red chromatophores. The yolk is further reduced and the egg has become a lighter green in colour. The embryo makes frequent muscular contractions. Abbreviation: *E* eye. Photograph courtesy of R. Clifford.

Figure 7.6.

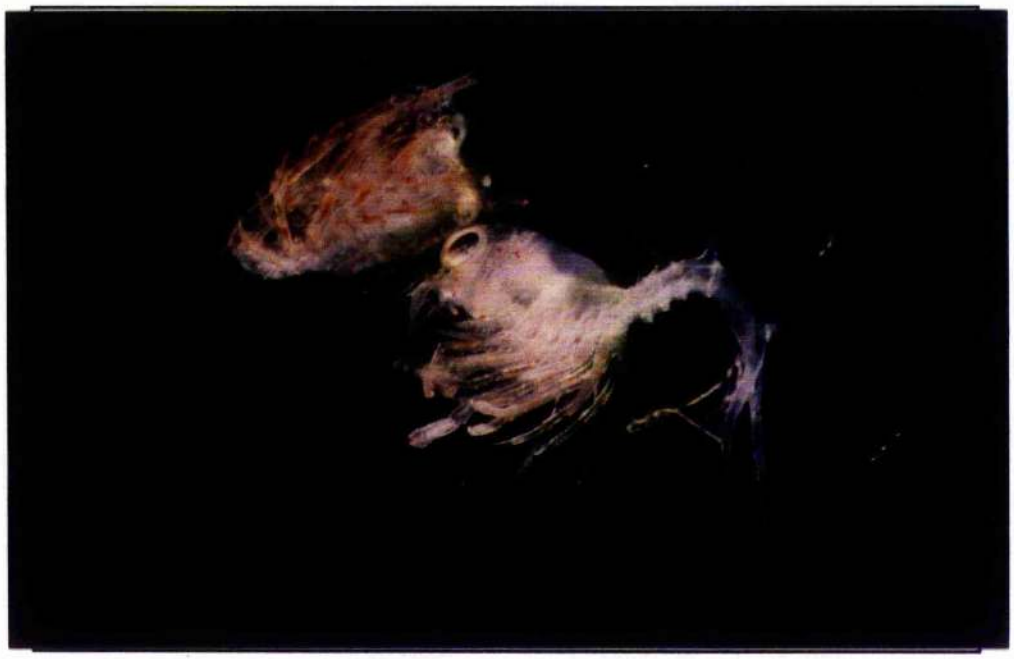
Unfixed, intact egg of *Nephrops norvegicus* at approximately E90%. The eye is very large and heavily pigmented with fully developed ommatidia. The thoracic appendages are well developed and display considerable movement, the embryo often gives violent muscular contractions. The chromatophores are present throughout the embryo, and give the eggs an orange appearance. The paired hepatopancreas are seen as darkened areas divided by the telson. The yolk has been almost totally absorbed by the hepatopancreas. Abbreviations: *E* eye.


E


E

Figure 7.7.

First zoea of *Nephrops norvegicus*. This is equivalent to the stage 1 larvae of *Homarus gammarus* which were demonstrated to be capable of synthesis and storage of CHH and GIH (Rotllant *et al*, 1992).



7.3.2 Preparation of total RNA from eggs of *Nephrops norvegicus*.

The modified method of Sambrook *et al* (1989) described in section 7.1.2 appeared at first to give better results, however, the total RNA obtained following the final LiCl precipitation, was purple in colour. As described in section 7.1.2, it was thought that the colour may be a natural dye and it was considered necessary to remove the colour before attempting cDNA synthesis. The Qiagen column did not successfully remove the colour. The spectrophotometric ratio of the sample at 260:280nm was 0.294 : 0.181. A value of 1.62 was obtained (2.0 = pure DNA, 1.8 = pure RNA, $A_{260}=1$ corresponds to $40\mu g.ml^{-1}$), the quantification of total RNA obtained from the Qiagen column was assessed as $0.6\mu g.\mu l^{-1}$ in 10µl. Synthesis of first strand cDNA was traced using incorporation of α -dCTP [P^{32}] following TCA precipitation. The amplification of CHH specific cDNA by PCR using the degenerated oligonucleotide primers (A gift from F. Van Herp, Nijmegen) described in Chapter 2, section 2.21, was unsuccessful.

7.3.3 ELISA of CHH and GIH on various stages of egg development.

When using the ELISA on the egg extracts, it was observed that the time required for the colour reaction to occur was considerably longer than for the same reaction that was adopted on the HPLC fractions, described in Chapter 4. Figure 4.10 demonstrates an ELISA carried out on the active HPLC fraction, 42, where a dilution gradient of the antigen was adopted. Figure 7.8 shows the same dilution gradient of the HPLC fraction, with a colour reaction time identical to the egg ELISA. The samples of 2, 1 and 0.5 sinus gland equivalents are all spectrophotometrically overloaded at 490nm, demonstrating a clear difference

between the ELISA carried out on the HPLC fraction and that carried out on the egg extracts. This will enable a comparison of the immunoreaction of the eggs with the CHH active HPLC fraction and allow an estimation of sinus gland concentration of antigen produced by comparison to the HPLC extract.

The use of anti-*Orconectes* CHH and anti-*Homarus* GIH antisera has enabled the detection of CHH and GIH immunopositivity within embryonic stages of *Nephrops norvegicus*. Figure 7.9 shows the percentage development of the eggs of *N. norvegicus* used as a sample for the detection of CHH immunoactivity and demonstrates that there is a significant increase of CHH immunopositivity after the eggs are 50% developed (Student t test = $p<0.01$; for Tukey's honesty significance test [HST] see table 7.2). Once the eggs are 90% developed there is further increase of CHH immunoactivity (Student t test = $p<0.001$; for Tukey's [HST] see table 7.2). When the 90% developed eggs are compared to the antigen binding assay (figure 7.8), it can be estimated that 20 eggs contain an immunoactive concentration of >0.125 but <0.25 sinus gland equivalents.

An increase of GIH immunoactivity can be observed during the development of the eggs tested with the anti-*Homarus* GIH antisera (figure 7.10). Again there is no significant increase of GIH immunoactivity until 50% development (Student t test = $p<0.01$; for Tukey's [HST] see table 7.2) and then a highly significant increase of immunoactivity occurs by the time the eggs are 90% developed (Student t test = $p<0.001$; for Tukey's [HST] see table 7.2). Figure legends under figures 7.9 and 7.10 give further details of the significant differences between the developmental stages of the eggs for both CHH and GIH immunoactivity. When comparing the results using the anti-*Orconectes* CHH and anti-*Homarus* GIH antisera, it is clear that there is a significantly increased immunoreaction when using the GIH antisera when compared to the CHH antisera. This result is discussed further below.

Figure 7.8.

ELISA was performed using the polyclonal rabbit anti-*Orconectes* CHH antisera on a dilution gradient of the HPLC purified fraction 42 that displayed immunoactivity to the above antisera (see chapter 4, figure 4.10). The ELISA colour reaction was stopped with 3M H_2SO_4 at the same time as the ELISA for the egg homogenates. The horizontal line above x axis represents the optimal absorbance at 490nm for the 90% developed egg homogenates and allows an estimation of sinus gland equivalents present in 20 *Nephrops norvegicus* eggs.

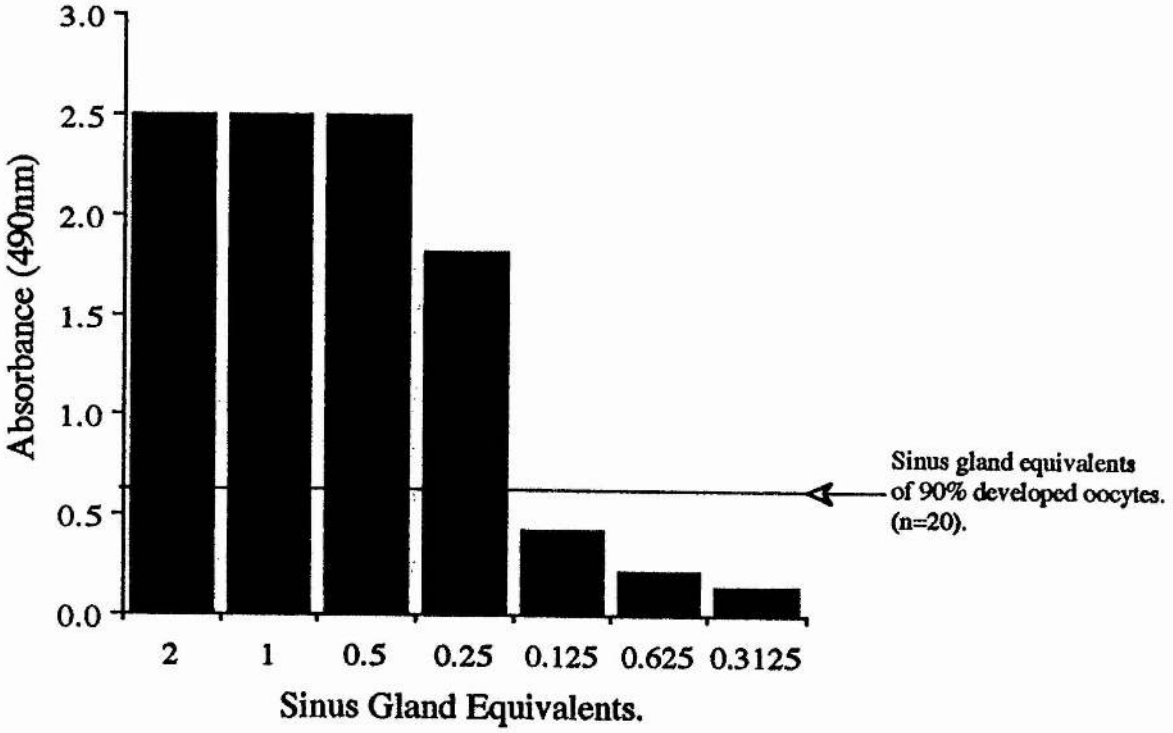
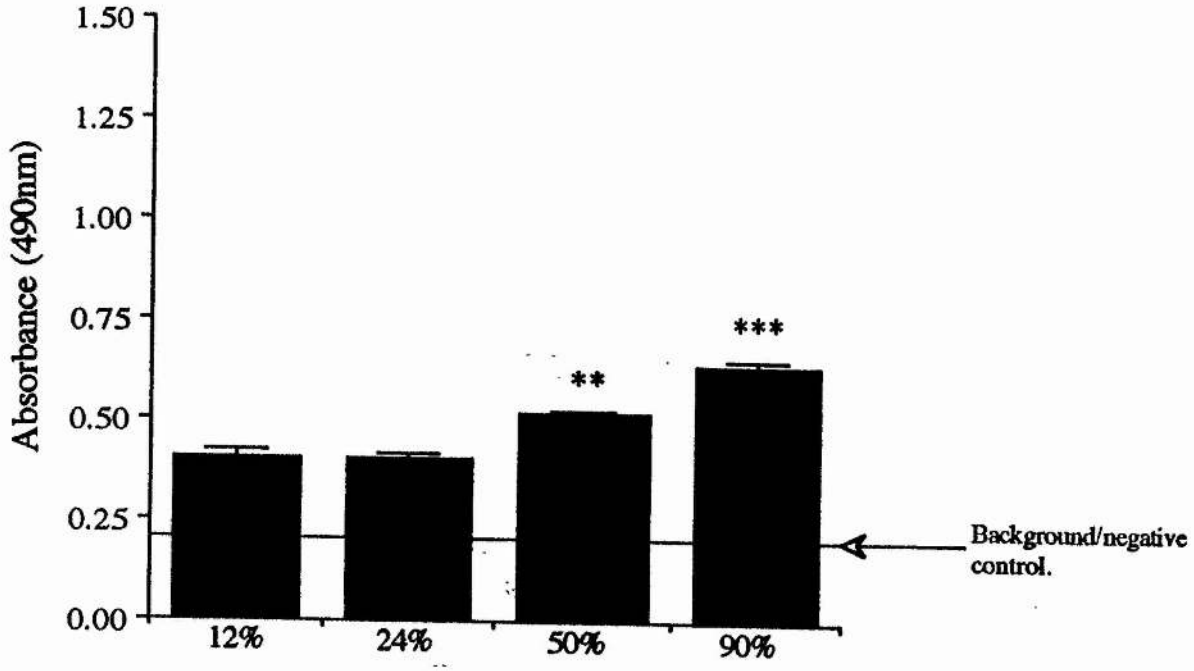

3.0
2.5
2.0
1.5
1.0
0.5
0.0
Absorbance (490nm)
2
1
0.5
0.25
0.125
0.625
0.3125
Sinus Gland Equivalents.
Sinus gland equivalents of 90% developed oocytes. (n=20).

Figure 7.9.

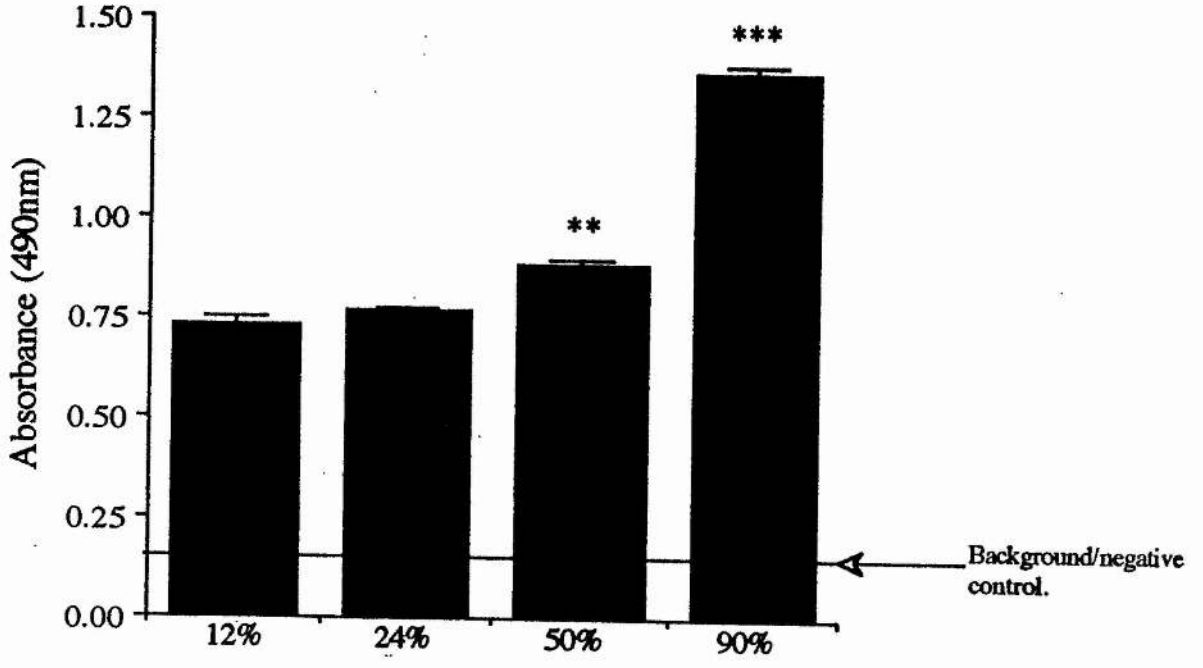
ELISA was performed using the polyclonal rabbit anti-*Orconectes* CHH antisera on homogenates of 20 *Nephrops norvegicus* eggs at varying percentages of egg development. Details of the ELISA protocol and antisera used are described in the text. The resulting optical density was recorded at 490nm. The horizontal line above x axis represents the optimal absorbance at 490nm for the PBS negative control. Values are represented as mean ± sem n=4. Significant differences were calculated using the Students t-test and are indicated by asterisks: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to the 12% developed eggs.

Figure 7.10.

ELISA was performed using the polyclonal rabbit anti-*Homarus* GIH antisera on homogenates of 20 *Nephrops norvegicus* eggs at varying percentages of egg development. Details of the ELISA protocol and antisera used are described in the text. The resulting optical density was recorded at 490nm. The horizontal line above x axis represents the optimal absorbance at 490nm for the PBS negative control. Values are represented as mean ± sem n=3. Significant differences were calculated using the Students t-test and are indicated by asterisks: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to the 12% developed eggs.



Percentage Egg Development.



Percentage Egg Development.

Table 7.2

Data describing Students t-test, ANOVA and Tukey's honesty significance test on variations of CHH and GIH immunoreactivity during the development of *Nephrops norvegicus* eggs.

```
MTB > prin c1-c4

 ROW  chh time  chh abs  gih time  gih abs

   1         1    0.460         1    0.697
   2         1    0.380         1    0.728
   3         1    0.411         1    0.770
   4         1    0.358         2    0.784
   5         2    0.429         2    0.746
   6         2    0.408         2    0.770
   7         2    0.365         3    0.899
   8         2    0.410         3    0.860
   9         3    0.502         3    0.899
  10         3    0.514         4    1.390
  11         3    0.535         4    1.376
  12         3    0.519         4    1.338
  13         4    0.641
  14         4    0.627
  15         4    0.674
  16         4    0.635

MTB > Oneway 'chh abs' 'chh time';
SUBC>   Tukey 5.

ANALYSIS OF VARIANCE ON chh abs
SOURCE     DF        SS        MS        F        p
chh time    3  0.159224  0.053075    64.34    0.000
ERROR      12  0.009899  0.000825
TOTAL      15  0.169123

                                    INDIVIDUAL 95 PCT CI'S FOR MEAN
                                    BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  ---+---------+---------+---------+---
     1      4   0.40225   0.04421  (--*--)
     2      4   0.40300   0.02704  (--*--)
     3      4   0.51750   0.01367            (--*--)
     4      4   0.64425   0.02065                      (--*---)
                                    ---+---------+---------+---------+---
POOLED STDEV =  0.02872             0.40      0.50      0.60      0.70

Tukey's pairwise comparisons

    Family error rate = 0.0500
Individual error rate = 0.0117

Critical value = 4.20

Intervals for (column level mean) - (row level mean)

               1         2         3

     2  -0.06106
         0.05956

     3  -0.17556  -0.17481
        -0.05494  -0.05419

     4  -0.30231  -0.30156  -0.18706
        -0.18169  -0.18094  -0.06644
```

```
MTB > Oneway 'gih abs' 'gih time';
SUBC>   Tukey 5.

ANALYSIS OF VARIANCE ON gih abs

SOURCE      DF        SS        MS        F        p
gih time     3  0.778598  0.259533   352.79    0.000
ERROR        8  0.005885  0.000736
TOTAL       11  0.784483

                                    INDIVIDUAL 95 PCT CI'S FOR MEAN
                                    BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  ------+---------+---------+---------+
     1      3    0.7317    0.0366  (-*)
     2      3    0.7667    0.0192    (*-)
     3      3    0.8860    0.0225         (-*-)
     4      3    1.3680    0.0269                                  (*-)
                                    ------+---------+---------+---------+
POOLED STDEV =    0.0271              0.80      1.00      1.20      1.40

Tukey's pairwise comparisons

   Family error rate = 0.0500
Individual error rate = 0.0126

Critical value = 4.53

Intervals for (column level mean) - (row level mean)

              1         2         3

     2  -0.10594
         0.03594

     3  -0.22527  -0.19027
        -0.08340  -0.04840

     4  -0.70727  -0.67227  -0.55294
        -0.56540  -0.53040  -0.41106
```

7.4 Discussion.

To date, there appears to be little detailed literature on the embryonic development of in *Nephrops norvegicus.* Most of the studies have been in the 1960's and 1970's and do not quantitatively stage the animal's development during embryogenesis or moult cycle (Figueiredo and Barraca, 1963; Farmer, 1974). In contrast, the embryonic development and physiology of *Homarus spp.* has been extensively investigated. Helluy and Beltz (1991) fully characterised the embryonic moult cycle and stage of *Homarus americanus*, categorising ten separate developmental stages from the early naupliar phase, through the metanaupliar stage and up to the first larval zoea. In addition, there has been considerable recent investigations on the neural development of embryonic *Homarus americanus* (Cole and Lang, 1980; Beltz and Kravitz, 1987, Beltz *et al*, 1990, Helluy and Beltz, 1990, Meier and Reichert, 1990). It has been suggested that characterisation of the metanaupliar moult cycle will provide information vital for the further investigation of neural, physiological and ecological aspects of embryonic development (Helluy and Beltz, 1991). A better understanding of the embryonic development of *N. norvegicus* will provide a basis for further embryological research.

The results of the growth curves obtained for *Nephrops norvegicus* egg development (data obtained by Clifford), match closely those obtained by earlier research (Farmer, 1974). Laboratory incubation at varying temperatures have indicated that a temperature increase of 10°C will approximately halve the period of incubation. The curves follow a similar line throughout development irrespective of temperature increase, which suggests that natural development occurs during artificial incubation periods. These preliminary investigations appear to indicate that day-length as well as temperature has an important role to play during embryonic development.

Embryogenesis in *Nephrops norvegicus* has been reviewed by Farmer (1975), and representative stages of development have been described by Fontaine and Warluzel (1969). To date, it appears that little further embryonic biology has been conducted on this species. It is proposed that in the light of recent studies such as that of Helluy and Beltz (1991), that a more detailed account of the embryonic development of *N. norvegicus* should be conducted. This is as especially prevalent as work by Beltz *et al* (1990) and Helluy and Beltz (1990) identified serotonin and proctolin immunoreactivity at various embryonic stages. Serotonin immunoactivity, studied as a marker for developing nervous structures, has been detected as early as E11% in *H. americanus*. By E50% immunoreactivity is identified throughout the embryonic nervous system, however, the appearance of proctolin immunoreactivity is relatively late and protracted when compared to serotonin (Beltz *et al*, 1989). The role of serotonin and proctolin in crustacean endocrinology has been discussed in Chapter 1 and Chapter 5. As serotonin and dopamine may be involved in the regulation of CHH synthesis and release, mediated by synaptic input of CHH axon ramifications in the medulla terminalis (Gorgels–Kallen, 1985; Van Herp and Kallen, 1991), it would suggest that the presence of these biogenic amines in the early developmental stages of *H. americanus* could indicate the production of CHH by the embryonic eyestalk neurosecretory structures.

The results described in section 7.1.3 of this chapter, demonstrate that CHH and indeed GIH immunoreaction does occur during the embryonic development of *Nephrops norvegicus*. At which stage of oogenesis CHH and GIH production is initiated is, as yet, unclear. However, an increase of each of the neuropeptides has been demonstrated after 50% development (figure 7.9 and 7.10). Once the embryo has reached late metanaupliar phase (approximately 90% of the total development) there is a significantly increased immunoactivity of CHH and GIH. All the egg stages show a significant increase of

immunoactivity when compared to the blank controls (Student t test = $p<0.001$), which suggests that immunopositive activity of the specific antisera to the egg extract is present throughout oogenesis. Alternatively, there is a considerable degree of non specific binding of the antisera to the egg extracts. Considering that haemolymph samples of *Nephrops* demonstrate extremely high levels of non specific binding to the extent that DAS-ELISA methods would be required to determine haemolymph CHH (Gorgels–Kallen and Voorter, 1985; see also Chapter 5), it seems that the latter explanation is the most likely. Unequivocal results could be obtained using the more specific DAS–ELISA method.

Figure 7.8 describes a direct ELISA of a serial dilution of the CHH active HPLC fraction number 42 that was not stopped and allowed to proceed for the same reaction time as that of egg extract. A comparison of CHH immunoactivity in the 90% developed eggs and the serial dilution of the active fraction suggests that the optical density of an ELISA reaction with 20 eggs of *Nephrops norvegicus* corresponds to a little over 0.125 sinus gland equivalents from the active HPLC fraction. Immunocytochemical investigations of the developing embryo may substantiate this result. This type of research on *Homarus gammarus* (Rotllant *et al*, 1992) demonstrated that CHH and GIH producing cell systems in the larval and post larval eyestalk are similar to those of adult lobster that were described by Kallen and Meusy (1989) and De Kleijn *et al* (1992), however, their number and size have been demonstrated to be lower in the larval stages. This reduction of size and number of neurosecretory cells in the larval stages of *H. gammarus*, would explain why the CHH immunopositivity in the developing *N. norvegicus* embryo is low when compared to the purified HPLC fraction.

It appears from the results in figures 7.9 and 7.10, that there is considerably greater GIH immunopositivity than that of CHH throughout all developmental stages of *Nephrops norvegicus*. In addition, the increases of

GIH production in the latter stages of oogenesis are significantly larger, suggesting a greater number of GIH producing cells than that of CHH. This is probably a misleading result. The number of cells that are CHH, GIH and CHH/GIH immunopositive in the larval stages of *Homarus*, have been shown to be approximately equal in number (Rotllant *et al*, 1992). If a similar cell distribution is observed in *N. norvegicus*, as one would expect, then this would cast doubt on the increased immunopositivity of GIH in the *N. norvegicus* egg extracts obtained in this study. It was noticed that the anti-*Homarus* GIH antisera, used to identify GIH immunoactivity in HPLC purified sinus gland extracts, was immunoactive to the same region that was CHH immunoactive (see figure 4.3.1). It is probable, therefore, that the anti-*Homarus* GIH antisera is reacting with both the GIH and the CHH antigen in the egg extract thus producing an artificially high immunoactive response.

The detection of CHH cDNA in the developing eggs would have supported the results obtained by the ELISA by indicating at which stage of maturation CHH synthesis in the embryonic tissues occurs. The method of total RNA preparation from the 50% developed eggs, that was used in this study resulted in a yield of $0.6\mu g.\mu l^{-1}$ of total RNA. The amplification of cDNA using the CHH specific primers in the PCR was, however, unsuccessful. It is not clear whether the unsuccessful priming was due to a poor mRNA yield as mRNA is only 2-3% of the total RNA, the degredation of the RNA sample during the extended purification procedure, or simply that in 50% developed eggs of *Nephrops norvegicus*, there is no expression of CHH. As the use of *in situ* hybridisation has demonstrated that the eyestalk of *Homarus gammarus* are endocrinologically active in stage I larvae (Rotllant *et al*, 1991), it would be interesting to determine if embryonic stages of *N. norvegicus* also have an active endocrine system. Results described here suggest that they have.

In conclusion, CHH and GIH immunoactivity can be identified in the eggs of *Nephrops* in which the embryo is only 50% developed, however, the identification of CHH cDNA, and thus, CHH expression could not be determined at this developmental stage. As the embryonic development approaches completion there is an increase in the production of both CHH and GIH which would suggest that neuropeptide production occurs as early as the metanaupliar larval stage of *Nephrops norvegicus*. The immunocytochemistry of the embryonic and larval stages of *N. norvegicus* is currently under investigation.

Chapter 8.

Discussion.

8.1 General Discussion.

The primary role of this thesis is to investigate crustacean hyperglycaemic hormone (CHH) in the Norway Lobster, *Nephrops norvegicus*. Information concerning this hormone is predominantly the result of research on *Carcinus maenas*, a brachyuran, and *Orconectes limosus, Astacus leptodactylus* and *Homarus americanus*, all of which are from the same phylogenetic grouping, the Astacidea. The biological significance of CHH isomerism is not fully understood. However, the presence of isoforms and the demonstration that in *H. americanus* their origins are from two different genes (Tensen *et al,* 1991b) suggests an explanation for the multifunctional role of CHH. CHH is involved in a number of regulatory process including the homeostatic control of glycogen metabolism (see Keller *et al,* 1985 for review), the stimulation of oocyte growth (Tensen *et al*, 1989), the release of digestive amylase (Sedlmeier, 1988), an involvement in the regulation of ecdysteroid synthesis (Webster, 1992; 1993) and is associated with effects resulting from physiological stress (Keller and Orth, 1990). The presence of CHH isomerism and its physiological significance is currently of great interest to many researchers. *N. norvegicus* is phylogenetically related to the Astacideans mentioned above, although, to date there has been little research into its endocrinology (see Chapter 1). Investigations of CHH in *N. norvegicus* has therefore, three major points of interest. Firstly, to obtain information concerning the endocrinology of the species, this being especially relevant, due to this species commercial importance to the European fishing industry. Secondly, to isolate, purify and characterise *N. norvegicus* CHH. This will enable a precise comparison of its structure and function to CHH from other crustaceans and thus determine if the high structural homology and isomerism that exists within other Astacideans, is maintained. Finally, the biological activity of CHH and its comparison to other phylogenetically related species, will provide information concerning the physiological adaptation of

crustaceans to differing habitats and environmental conditions. This last point may provide an explanation for any differences between CHH structure and function from that of other Astacideans.

The first part of this thesis deals with the elucidation of the biochemical nature of CHH. Initially, the identification of the sinus glands in *Nephrops norvegicus* and the action that injected crude sinus gland extracts had on the haemolymph glucose of this species was determined. These preliminary experiments determined the parameter of the response of *N. norvegicus* to sinus gland extract injection. It was demonstrated that 0.5 sinus gland equivalents evoked a maximal increase of glycaemia and that increases in sinus gland concentration appeared to have an inhibitory affect on glucose production. Furthermore, a significant increase of glycaemia could be induced by the injection of as little as 0.25 sinus gland equivalents. Dose response studies in other species have indicated a greater sensitivity to sinus gland extracts. Juvenile *Homarus americanus* display significant hyperglycaemia with 0.1 sinus gland equivalents (Soyez *et al*, 1990), while 0.05 sinus gland equivalents from *H. americanus*, induced a glucose increase in *Orconectes limosus* (Tensen, 1991). The pervasiveness of injected sinus gland extracts indicated that a maximal increase of glycaemia was obtained after five hours and that the levels began to drop following this. The exaggerated maintenance of hyperglycaemia is possibly due to the significant role of stress hyperglycaemia. Repeated hourly sampling appears to induce hyperglycaemia in *N. norvegicus* and this stress effect may have contributed to the apparent extension of hyperglycaemia following sinus gland injection. Saline injections into the animal show a nonsignificant increase in glucose after a single sampling time of 3 hours (see Chapter 3). The effects of stress released CHH could have been negated had control animals undergone eyestalk ablation, however, experiments involving this surgery were not very successful. The mortality of *N. norvegicus* in the aquarium is extremely high and a considerable number of animals is required to obtain a steady

population of animals for experimentation. The action of eyestalk ablation is highly stressful and the mortality can be as high as 50% even following careful surgical technique (see Chapter 2). As the bioassay involves only two injections three hours apart, the effects of stress-related hyperglycaemia are less marked.

The use of a two step HPLC fractionation of sinus gland extracts, enabled the separation of peptides depending on their hydrophobicity. Prior to this Sep Pak column purification enabled the identification of the solvent concentration which eluted the majority of hyperglycaemic activity that was contained within the sinus gland extracts. These data enabled the most efficient solvent gradient possible for the HPLC purification step (see Chapter 4).

The two different HPLC purification procedures yielded slightly differing results. The propanol gradient resulted in a chromatogram where a region of activity to the polyclonal rabbit anti-*Orconectes* CHH extended over only seven fractions. There were other smaller areas of immunoactivity, although these could be due to cross-reactivity with other eyestalk neuropeptides, namely GIH or MIH. The main bioactive peak was asymmetric and could be a region of CHH isomerism. The HPLC purification protocol that utilised an acetonitrile gradient produced a chromatogram showing immunoactivity extending over more than 20 fractions, however, the majority of the activity was associated with three peaks. These peaks were separated with a further HPLC step and although only two (peak I and peak II) were capable of producing an immunoactive response, only peak I showed a significant increase of haemolymph glucose in both the heterologous and homologous bioassay. The use of SDS-PAGE indicates that these two peptides may have slightly different molecular masses, although the resolution of the gels is not capable of clearly distinguishing between them. The determination of molecular mass of each peak using FAB/MS will give an accurate indication of the size of each of the peptides. It is of interest that peak I showed immunoactivity to the polyclonal guinea pig anti-*Homarus* GIH antiserum and the polyclonal rabbit anti-*Orconectes*

CHH antiserum. The possibility exists that the antisera are non-specific, however, it would be interesting to bioassay this peptide for GIH activity. It is clear that the isolation and purification of CHH from *Nephrops norvegicus* is not complete and many questions remained to be answered. For example, it is not clear if CHH isomerism exists in this species. Better separation of the peaks I, II and III would enable greater information to be obtained concerning their activity. The immunoactivity has only been assessed on the HPLC fractions, however, the use of immunoblotting would provide greater support to the HPLC results while indicating the level of purity of each fraction. The anti-GIH activity warrants further investigation, not only as described above, on those fractions that displayed immunoactivity to both antisera, but to peaks IV and V. Additionally, the use of an antibody specific to MIH may help to elucidate the questions concerning CHH isomerism. Until an amino acid sequence has been obtained, it is not possible to make definite statements concerning the purification of CHH from the sinus glands of *N. norvegicus*. The use of the two different antisera raised against CHH in *Orconectes limosus* and GIH in *Homarus americanus*, indicates the inter-specificity of each of the antisera, while the use of *Pacifastacus leniusculus* in a heterologous bioassay demonstrates the inter specific action of CHH.

An investigation into the physiological significance of CHH in *Nephrops norvegicus*, was carried out and is described in Chapter 5. The effects of physiological stress and altered environmental conditions on the production of CHH and associated hyperglycaemia has been investigated in a number of crustacean species (see Chapters 1, 3 and 5), however, the understanding of the effects of these conditions is far from being understood. The effects of hypoxia on the physiology of aquatic invertebrates and the range of responses that they utilise to counteract declining oxygen tensions has been reviewed by Herreid (1980). The occurrence of severe oxygen deficiencies in the waters of the Kattegat, a stretch of water between Denmark and Sweden, has been caused by excessive eutrophication, high incidence

of phytoplanktonic blooms and hydrographical factors (Baden *et al*, 1990). These adverse conditions worsen towards the latter part of the year and ultimately have a detrimental effect on the benthic macro- and mega-fauna and on demersal fish (Rosenberg, 1985; Hagerman and Baden, 1988; Rosenberg and Loo, 1988; Pihl, 1989). Due to its relatively sedentary life, *Nephrops norvegicus* is one of the most severely effected species. The oxygen concentration experienced by *N. norvegicus* in the burrow is probably lower than that on the substrate surface (Atkinson and Taylor, 1988) and this induces an emergent behaviour discussed in chapter 1 and 5.

Apart from the emergent behaviour, the animal responds to this physiological stress by a number of other behavioural and physiological adaptations. It may lift its body off the substrate surface and maintain a "tiptoe" posture until it becomes moribund (Baden *et al*, 1990; Smullen, pers. observ.) and may increase the beat of both its pleopods and scaphognathite in order to increase the circulation of water around and within the body (Hagerman and Uglow, 1985). When exposed to severe hypoxia, there is an initial increase of haemocyanin concentration in the blood, although these decrease rapidly if the conditions prevail (Baden *et al*, 1990; Hagerman *et al*, 1990). This catabolism of haemocyanin under severe hypoxia is the animals "last resort". Glycogen has been shown to be preferentially mobilised from the hepatopancreas to provide an energy source by breakdown into lactic acid (Schmidt-Nielsen, 1975). It has been speculated that CHH adopts a globular structure (Kegel *et al*, 1989; Tensen, 1991) with a hydrophobic tail. This may interact with protein carriers, of which haemocyanin may be one (Kallen *et al*, 1990). The initial increase of haemocyanin in response to hypoxia may therefore provide the increased measurements of CHH in the haemolymph. Likewise the utilisation of the carrier due to long term exposure to hypoxia may effectively reduce the efficiency of CHH distribution to the target tissues. Results obtained by Smullen (unpublished) demonstrated an increase of lactic acid in the haemolymph of *N. norvegicus* when subjected to severe hypoxia. Furthermore, increases of circulating

glucose occurred after one hour and hyperglycaemia was at a maximum after two hours (see Chapter 5). Over the proceeding ten hours the glucose levels decreased to similar concentrations recorded at the start of experimentation. This is in direct contrast to the response of *Orconectes limosus* to severe hypoxia (Keller and Orth, 1990). The levels of glucose did not decline and severe hyperglycaemia occured after 12 hours of exposure to the low oxygen tensions. Although there was no increase of glucose during the first hour, there was a high concentration of CHH in the haemolymph, which as hyperglycaemia occured, decreased back to initial concentrations. It was not possible to measure CHH levels in *N. norvegicus*, however, the hyperglycaemia observed is probably the mobilisation of energy supplies described by Schmidt-Nielsen (1975). Work by Santos and Keller (1992) suggested that during hypoxia CHH increased the substrate availability allowing glycolysis to proceed at a higher rate.

Nephrops norvegicus are capable of withstanding oxygen tensions as low as 12% for nearly three weeks (Baden *et al*, 1990) and therefore, it appears that they are able to utilise glycogen reserves enabling long term adaptation to exposure to severe hypoxia. An understanding of the endocrinology of *N. norvegicus* during these periods of stress are becoming ever more prevalent. Increased eutrophication of coastal waters may destroy an existing population of *N. norvegicus*. However, as juvenile and egg bearing females, which under normal conditions would remain inside the burrow, are forced out due to reduced oxygen tensions, their catchability increases dramatically (Bagge and Munch-Petersen, 1979). Therefore, eutrophication, by creating hypoxic conditions, ultimately effects future populations of *N. norvegicus*.

The effect of diurnality on the production of CHH and glucose rhythms has been investigated in both *Orconectes limosus* (Hamann, 1974; Kallen *et al*, 1990) and *Astacus leptodactylus* (Gorgels-Kallen and Voorter, 1985; Kallen *et al*, 1988). The crayfish shows a circadian rhythmicity with a peak of CHH production

occurring at the onset of darkness, which results in an immediate rise of CHH in the haemolymph and in hyperglycaemia 2 to 4 hours later. The same process is repeated, although to a lesser extent, at the start of the light phase when CHH production occurs a few hours prior to the start of the light period. There is also a corresponding circadian rhythm of synthetic activity in the CHH producing cells in the eyestalk (Gorgels-Kallen and Voorter; 1985). In addition, there is a peak of CHH mRNA activity during the light phase approximately 5 hours before the onset of the dark period (Tensen, 1991), while the lowest levels were observed at the start of the light phase.

Experiments described in Chapter 5 investigated whether any circadian rhythm of glucose exists in *Nephrops norvegicus*. As discussed in Chapter 1 and 5, the environmental range of the species extends over considerable depth. Previous work has demonstrated that the movements of *N. norvegicus* out of their burrows corresponded to the change in light intensity (Farmer; 1974; Aréchiga and Atkinson, 1975; Atkinson and Naylor, 1976; Chapman and Howard, 1979), therefore it was considered that any increases of haemolymph glucose would occur during or just before these periods. The results obtained and described in Chapter 5 suggested that the sample population consisted of two sets of animals; those that demonstrated a circadian rhythm of glucose and those that did not. The animals that displayed a rhythm of glucose, experienced increases of circulating glucose, the peaks of which appeared to correspond to these periods of activity described above. However, the other individuals experienced little variation of glucose, at lower concentrations than those that displayed a glycaemia rhythm. This unexpected result is discussed in detail in Chapter 5, however, since it was not possible to measure haemolymph CHH levels the true nature of this variation is unclear. As *N. norvegicus* are naturally abundant over a vast range of depths, the influence of day length may vary in importance when compared to the other Astacuran species investigated that may

only be found in shallow waters. Further experiments to investigate this phenomenon are described later.

Molecular biological techniques were adopted, primarily to isolate and characterise cDNA clones that encode CHH from *Nephrops norvegicus* (see Chapter 6). The Polymerase Chain Reaction (PCR) was used in combination with degenerated oligonucleotide primers specific to regions of the CHH sequence of *Homarus americanus* (Tensen, 1991). The subsequent cDNA amplification from total eyestalk RNA of *N. norvegicus* produced two PCR products, a 100bp product utilising primers 1 and 2, and a 230 bp product with primers 1 and 3. Although the cloning and sequencing of the products was not successful, useful information concerning the structure of *Nephrops* CHH had been obtained. There must be considerable homology of the nucleotide sequence of *Homarus* CHH with that of *Nephrops* CHH, however, there may be less similarity at the 3' antisence end of the sequence. Furthermore, as this method is capable of detection and amplification of a single gene copy of CHH, it is hoped to be able to detect the synthesis of CHH qualitatively and semi-quantitatively during the physiological experiments described earlier. It will therefore provide subsequent information concerning the mechanisms of the endocrinology of *Nephrops* under these extreme conditions.

In the final chapter, the endocrinological mechanisms of embryogenesis were investigated by the use of immunobiological and molecular biological techniques described in Chapters 4 and 6. The developmental and morphological changes during embryogenesis were identified and described, while the rate of embryonic development under varying light and temperature regimes were monitored. At various stages of development, the eggs were assessed for CHH and GIH production by the use of polyclonal rabbit anti-*Orconectes* CHH antisera and polyclonal guinea pig anti-*Homarus* GIH antisera. There was a prevailing immunoactivity throughout the development of the eggs, although it is considered that this may be due to the non-specific binding of the antisera. However, an

increase of immunoactivity to both CHH and GIH antisera was demonstrated in 50% developed eggs and a further increase occurred in eggs which were 90% developed. Unfortunately, it was not possible to detect any synthetic activity of the CHH producing cells in the 50% developed embryos. It is possible however that the RNA preparation from the eggs was unsuitable due to the high quantities of lipid and carbohydrate present and in future the removal of the developing embryo from the egg capsule may provide better results. The photograph shown in Chapter 7 demonstrate that at 50% development, the eye is considerably large and by 90% development the structures of the eye, such as the ommatidia are visible. At what stage the eyestalk neurosecretory structures become active is not clear, however, these results suggest the MTGXO-SG complex is endocrinologically active at the late metanaupliar stage, prior to hatching. The significance of the production of CHH and GIH at this stage is open to speculation, but by late embryonic development, much of the yolk has been used as an energy supply by the embryo. The first caeca of the paired hepatopancreas are observed by 50% development in both *Homarus americanus* (Helluy and Beltz, 1991) and in *Nephrops norvegicus* (Chapter 7) and by 90-100% development, the yolk is almost entirely absorbed and is attached to the hepatopancreas. The development of the hepatopancreas concurrently with embryonic eyestalk structures suggests that the increase of CHH production/immunoactivity may be acting on the hepatopancreas and the glycogen stores to provide energy for later embryogenic changes. The increase production of GIH may be necessary to inhibit the reproductive development of the embryo by effectively preventing vitellogenin deposition.

The experiments described in this thesis demonstrate that there is considerable cross-reactivity of CHH activity between species that are phylogenetically related. Evidence for this cross reactivity is provided by the use of heterologous bioassay in Chapters 3 and 4 while antisera specific to CHH from *Orconectes limosus* and *Homarus americanus*, demonstrate species non-specific

immunoactivity. Finally, the use of PCR using the *Homarus* primers indicates a further point of endocrine homology between the species. There are many areas of the endocrinology of *Nephrops norvegicus* that remain unexplained. The fact that the species is found in quite different biotopes to *Carcinus maenas, H. americanus, O. limosus* and *Astacus leptodactylus*, from which the majority of the information concerning crustacean endocrinology is derived, may provide some useful comparisons. Furthermore, the range of habitats in which *N. norvegicus* can be found may show within species variation of physiological and endocrinological systems.

8.2 Future experiments.

The purification of CHH from *Nephrops norvegicus* is not complete. The lack or presence of CHH isoforms could be confirmed by using immunoblotting and FAB/MS techniques. Ultimately an amino acid sequence may be obtained and compared to a nucleotide sequence. The raising of an anti-*Nephrops* CHH would perhaps provide a more sensitive assay for immunological investigations, while oligonucleotide primers specific to regions of the *Nephrops* CHH would enable the accurate determination of mRNA in the species under varying experimental conditions.

In order to understand the endocrine system of *Nephrops norvegicus* under varying environmental condition, the detection of circulating levels of CHH would provide valuable information, as would the identification of mRNA levels in order to determine how the synthesis of CHH is effected by hypoxia. As the action of CHH is to stimulate the production of cyclic nucleotides, their measurement of concentration in target tissue could elucidate the action of CHH during these stresses. Likewise, the use of immunological and molecular biological techniques used in the experiments reported in this thesis, may indicate that the action of the

endocrine system differs between *N. norvegicus* populations that are in contrasting water depths and therefore under contrasting light/dark regimes.

Finally, the role of the endocrine system during embryogenesis is poorly understood. The development of the eggs, larvae and reproductive systems in Crustacea, have currently been areas of increased interest. Immunocytochemical investigations into the development of CHH and GIH in the embryonic stages of *Nephrops norvegicus* are currently under investigation, and it is hoped to identify at which point during the eggs development the synthesis of CHH occurs.

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Appendix 1. Amino Acid Abbreviations

One-letter	Three-letter	Name
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine